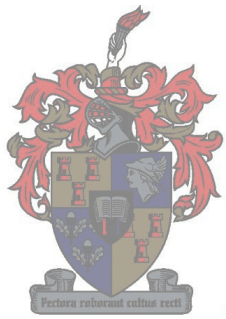
The effects of the synthetic strigolactone GR24 on *Arabidopsis thaliana* callus culture

By

Ntombizanele Thobela Mdodana

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Plant Biotechnology at the University of Stellenbosch



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Supervisor: Dr, Paul N Hills
Co-supervisor: Prof, Jens Kossmann
Faculty of Natural Sciences
Department of Genetics
Institute for Plant Biotechnology

December 2012

Declaration

I the undersigned, hereby declare that the work contained in this thesis is my own work (Unless to the extent explicitly otherwise stated) and that I have not previously, in its entirety or in part submitted it at any university for a degree.

Signed…….. Date……..

Summary

Plant growth promoting substances (PGPS) are emerging as useful tools in the investigation of important plant growth traits. Two PGPS, smoke-water derived from burning plant material and a synthetic strigolactone analogue, GR24, have been reported to regulate a wide variety of developmental and growth processes in plants. These PGPS are beginning to receive considerable attention in the area of improving plant biomass yield and production. Variation in growth between plants is a major impediment towards the complete understanding of the intrinsic processes that control biomass production. Callus cultures of the model plant *Arabidopsis thaliana* could overcome some of these hindrances. However, the suitability of these callus cultures as a model system for plant biomass production must be established first. This study aimed at using *A. thaliana* callus cultures as a platform to study the plant growth promoting activities of smoke-water and GR24.

The first part of this study was conducted to develop an optimal protocol for inducing *A. thaliana* callus formation. Wild-type *A. thaliana* Col-O, as well as strigolactone deficient and insensitive mutants (*max1-1*, *max2-1*, *max2-2*, *max3-9* and *max4-1*) were cultured for callus induction. Hypocotyl and leaf explants were cultured onto MS media supplemented with different hormone concentrations of 2,4-D and kinetin (2:2 mg/L 2,4-D:kinetin and 0.5:0.05 mg/L 2,4-D:kinetin). Both media proved suitable for callus induction of all genotypes, with *max1-1* showing the highest efficiency (83.33% and 92.22%) of callus induction. Calli were then used as a platform for future investigations into the effects of smoke-water and GR24.

Secondly, this study examined the effects of smoke-water and GR24 on wild-type *A. thaliana* Col-O callus. Basic physiological studies were conducted to determine if these two compounds would positively affect callus growth, as was shown in previous studies using whole plants. Calli cultivated on MS media containing the two different hormone concentrations were transferred onto the same fresh MS medium, supplemented with either smoke-water or GR24. Growth promotion by smoke-water and GR24 in calli was characterized by a significantly increased mass (biomass). Calli were additionally transferred onto MS medium containing either auxin only or

kinetin only and supplemented with GR24 or smoke-water. In the auxin only system, increased mass was recorded for both GR24 and smoke-water treatments, while these two compounds seemed to reduce growth in the kinetin only system. The positive growth stimulatory effect observed for the auxin only system could be attributed to the synergistic relationship between auxin and strigolactones, whilst the reduced mass in the latter system could be due to the antagonistic interaction between strigolactones and cytokinins.

Finally, this study has discovered a dual role of strigolactones in biomass accumulation and adventitious root formation for *Arabidopsis thaliana* callus. On an auxin- and cytokinin-free MS medium supplemented with GR24, calli of *Arabidopsis thaliana* strigolactone deficient mutants (*max1-1* and *max4-1*) and the wild-type Col-O, but not the strigolactone response mutant (*max2-2*), showed enhanced biomass accumulation. In addition to this, the *max4-1* mutant and wild-type Col-O demonstrated enhanced adventitious rooting, which was not apparent in *max2-2*. Together these data suggested that the biomass accumulation and the adventitious rooting activities of GR24 in *Arabidopsis thaliana* calli are controlled in a MAX2-dependent manner. The interaction between strigolactone, auxin and cytokinin signalling pathways in regulating these responses appears to be complex. Gene expression profiling showed regulation of stress-related genes such as B-box transcription factors, *CALCINEURIN B-LIKE* and *RAP4.2* Genes encoding hormones associated with stress (ABA, ethylene) and defence mechanisms (JA) were up-regulated. Expression of stress related genes indicated clues on some kind of stress mediation that might be involved during the regulation of the rhizogenic response. Conversely, smoke-water treatment could not enhance the biomass of the calli and nor could it induce adventitious rooting in the absence of auxin and cytokinin. This observation strongly emphasized the distinct roles of these two compounds, as well as the importance of the interaction and ratio of auxin and cytokinin in callus growth.
This study has demonstrated a novel role of strigolactones in plant growth and development, i.e. enhancement of biomass production in callus cultures. Secondly the enhanced adventitious rooting ability is in agreement with recently published literature on the role of strigolactones in regulating root architecture. *In vitro* callus production is advantageous to plant sciences. It creates an opportunity for increasing

plant material for cultivation and offers the use of cell cultures that accurately mimic specific growth responses. It could greatly contribute to the study of intricate regulatory and signalling pathways responsible for growth and development in plants. Because the regulation of plant biomass production is very complex and the molecular mechanisms underlying the process remain elusive, it is of paramount importance that further work be done in order to gain more in-depth insights and understanding of this aspect and subsequently improve efficiency and returns when applying biotechnology tools on commercially important crop plants.

Opsomming

Verbindings wat plantgroei bevorder (PGBV) het as nuttige alternatief ontstaan om plant groei te ondersoek. Rook-water, afkomstig van verbrande plant material, en ‘n sintetiese strigolaktoon analoog, GR24, wat ‘n α, β-onversadigde furanoon funksionele groep in gemeen het, is vir die regulering van ‘n wye verskeidenheid ontwikkelings- en groei prosesse in plante verantwoordelik. Tans ontvang hierdie PGBVs aansienlik aandag in die area van die verbetering van plant biomassa opbrengs en -produksie. Die variasie in groei tussen plante is ‘n groot hindernis om die intrinsieke prosesse wat biomass produksie beheer, volledige te verstaan. Deur gebruik te maak van kallus kulture van die model plant *Arabidopsis thaliana* kan van hierdie hindernisse oorkom word. Tog moet die geskiktheid van kallus kulture as ‘n model sisteem vir plant groei biomass produksie eers gevestig word. Die doel van hierdie studie was om *A. thaliana* kallus kulture as ‘n platform vir die studie van die plantgroei bevorderingsaktiwiteite van rook-water en GR24 te gebruik.

Die eerste deel van die studie is uitgevoer ten einde ‘n optimale protokol vir die induksie van *A. thaliana* kallus produksie te ontwikkel. Wilde tipe Col-0, asook strigolaktoon afwesige en onsensitiewe mutante (*max1-1*, *max2-1*, *max2-2*, *max3-9* en *max4-1*) is vir kallus induksie gekultiveer. Hipokotiel en blaar eksplante is op MS medium wat verskillende hormoon konsentrasies van 2,4-D en kinetien (2:2 mg/L 2,4-D:kinetien en 0.5:0.05 mg/L 2,4-D:kinetien) bevat, oorgedra. Beide media was geskik vir kallus induksie van al die genotipes, met *max1-1* wat die hoogste effektiwiteit (83.33% en 92.22%) van kallus induksie getoon het. Kalli is daarna as ‘n platform vir toekomstige navorsing i.v.m die effek van rook-water en GR24 gebruik.
Tweedens ondersoek die studie die effek van rook-water en GR24 op wilde tipe Col-0 kallus. Basiese fisiologiese studies is uitgevoer om te bepaal of die twee verbindings ‘n positiewe effek op kallus groei toon soos aangedui in vorige studies waar intakte plante gebruik is. Kallus wat op MS medium wat die twee verskillende hormoon konsentrasies bevat gekultiveer was, is op dieselfde vars MS medium, wat addisioneel óf rook-water óf GR24 bevat, oorgedra. Die stimulering van groei van kalli deur rook-water en GR24 is deur ‘n merkwaardige toename in massa (biomassa) gekenmerk.

Kallus is additioneel op MS medium wat slegs óf ouksien óf kinetin bevat (gekombineer met GR24 of rook-water behandeling), oorgedra. In die sisteem waar slegs ouksien toegedien is, is ‘n toename in massa waargeneem vir beide GR24 en rook-water behandelinge. In teenstelling hiermee, het die twee verbindings in die sisteem waar slegs kinetin toegedien is, ‘n vermindering in groei meegebring. Die positiewe groei stimulerende effek wat waargeneem is vir die sisteem waar slegs ouksien toegedien is, kan toegedra word aan die sinergistiese verhouding tussen die ouksien en strigolaktone; terwyl die verlaagde massa in die laasgenoemde sisteem aan die antagonistiese interaksie tussen strigolaktone en sitokiniene toegedra kan word.

Laastens het hierdie studie het ‘n gelyktydige rol van strigolaktone vir biomassa akkumulasie en bywortelvorming in *Arabidopsis thaliana* kallus ontdek. Kallus van *A. thaliana* strigolaktoon afwesige mutante (*max1-1* en *max4-1*) en die wilde tipe Col-0 (maar nie die strigolaktoon reagerende mutant (*max2-2*) het op ‘n ouksien en sitokinien vrye MS medium wat GR24 bevat ‘n verhoogde biomassa akkumulasie getoon. Die *max4-1* mutant en wilde tipe Col-0 het verhoogde bywortelvorming getoon, wat nie so opmerklik by *max2-2* was nie. Hierdie data het tesame voorgestel dat die biomassa akkumulasie en die bywortelvormingsaktiwiteite van GR24 in *Arabidopsis thaliana* kallus op ‘n MAX2-afhanklike wyse beheer word. Die interaksie tussen strigolaktoon, ouksien en sitokinien sein transduksie paaie vir die regulering van hierdie reaksies blyk kompleks te wees. Die geen uitdrukkingsprofiel het die regulering van stres verwante gene soos B-boks transkripsie faktore, *CALCINEURIN B-LIKE* en *RAP4.2,* getoon. Gene wat vir hormone wat aan stres (ABA, etileen) en verdedigingsmeganismes (JA) verwant is, is opgereguleer. Die uitdrukking van stress verwante gene dui op tekens van ‘n ander tipe stres bemiddeling wat dalk by die regulering van die risogeniese reaksie betrokke kan wees. In teenstelling, rook water behandeling kon nie die kallus biomassa verhoog nie en dit kon ook nie die bywortelingvorming in die afwesigheid van ouksien en sitokiniene induseer nie. Hierdie waarneming is ‘n sterk bevestiging vir die uitsonderlike rol van die twee verbindings, asook die belang van die interaksie en verhouding van ouksien en sitokinine vir die groei van kallus.

Hierdie studie toon op ‘n nuwe rol van strigolaktoon in plant groei en ontwikkeling, d.w.s die verhoogde biomassa produksie in kallus kulture. Tweedens, die verhoogde bywortelvormingsvermoë is in ooreenstemming met literatuur wat onlangs gepubliseer is i.v.m die rol van strigolaktone in die regulering van wortel argitektuur. Die *in vitro* produksie van kallus is voordelig in plant wetenskappe. Dit skep ‘n geleentheid vir die vermeerdering van plant materiaal vir kultivering en bied die gebruik van selkulture wat spesifieke groei reaksies op ‘n merkwaardige wyse akkuraat namaak. Dit kan grootliks bydra tot die studie van die delikate regulatoriese en sein transduksie paaie wat vir groei en ontwikkeling van plante verantwoordelik is. Aangesien die regulering van plant biomassa produksie baie kompleks is en die molekulêre meganismes vir die proses onbekend bly is dit van grootskaalse belang dat meer werk gedoen word om ‘n meer in diepte insig en kennis van die aspekte en gevolglike verbetering van effektiwiteit en wins te kry deur die toepassing van biotegnologiese metodes op die gewas plante wat van kommersiêle belang is.

Dedication

To God be the glory, for the wonderful things he has done. Ebenezer my father, for you have brought me this far. This thesis is dedicated to the author and the creator of the universe, the Lord God Almighty and to my mother, Nomzi Mdodana.

Acknowledgements

I would greatly like to thank the support, help and advice given to me by my supervisor Dr Paul Hills during the course of this study.

I will forever be grateful for the opportunity given to me by Prof Jens Kossmann and the bank of knowledge imparted unto me for all my years of study at IPB.

I greatly appreciate the assistance of Mr Hannibal Musarurwa with statistics

My sincere gratitude goes to Stephan Ferreira, Riaan de Witt and Charl King for the microarray experiments and data analysis.

The molecular analysis would not have been complete without the help of Mr Sandile Ndimande, “ndiyabonga bhuti”.

I am grateful to the Plant Growth Promoting Substances group members; I also want to thank IPB staff and students for their support whilst embarking on this journey.

Special thanks goes to the body of Christ: we have together agreed, and it has been established by our father in heaven who makes all things possible, Jam 5:16; 2Cor 1:8-11; Rom 15:30; thank you all for prayers, love and encouragement. Amen.

I would like to express my deep sense of appreciation to my mother. Ukwenzile konke owawukuthembisile, mhla kungekuhle, qhawekazi. Ngazibini Rhadie, uThixo akusikelele. Amen.

Special thanks to my siblings for all their encouragement and for making me see the end from the beginning. Isandla sihlamba esinye, nangamso booValela.

Sis Khalie, it was too soon, you have a special place on each page of this thesis, may your soul rest in peace maValela

I am greatful to the National Research Foundation for financial support during my studies.

Above all I would like to thank my personal saviour, the Lord Jesus Christ, for keeping me through and for making this study a success, and thank you for believing in me Father.

Thesis Structure

This thesis is a compilation of six chapters. The document is structured as follows:-

Chapter 1

General introduction

Provides a general introduction and background to what plant biomass is, why it is important to study plant biomass production and briefly highlights some methods that have been used by researchers to manipulate plant biomass. Additionally, it includes some interesting plant growth regulators that can promote plant growth besides the classical plant hormones and consequently asking the question why biomass manipulation is important to investigate.

Chapter 2

Plant growth promotion

Is a review of current literature available on plant growth regulation. It provides an overview of what plant growth regulators are, their importance in plant growth and development and the different classification according to their functions. Thereafter, strigolactones, the newly discovered phytohormones that influence diverse aspects of plant growth are reviewed. Strigolactones are described based on their structure and the different functions that they have on plant growth and development. Additionally, aqueous smoke and its effects on plant growth is described. Furthermore, common features between strigolactones and KAR_1, the active constituent in smoke are highlighted. Moreover, the importance of using tissue culture as a scientific tool and the advantages of utilizing *Arabidopsis thaliana* callus cultures as model system have been described.

Chapter 3

In vitro* callus induction of *Arabidopsis thaliana

This chapter focusses on developing a protocol for rapid callus initiation of *Arabidopsis thaliana* wild-type and *more axillary growth* mutants. Explants were

cultured onto MS media supplemented with two different concentrations of 2,4-D and kinetin. Thus a suitable platform for studying the effects of strigolactones and smoke-solution in cell cultures was created.

Chapter 4

The effects of strigolactones and smoke in *Arabidopsis thaliana* callus cultures

Here the growth promoting properties of strigolactones and smoke were investigated, paying particular attention to their physiological effects on *Arabidopsis thaliana* callus cultures, in light of the structural resemblance between these chemichals.

Chapter 5

Strigolactones promote adventitious root in *Arabidopsis thaliana*

This chapter demonstrated the ability of strigolactones to promote adventitious root formation in *Arabidopsis thaliana* callus cultures. Strigolactone-deficient and response mutants were analysed to determine if the root stimulating function of GR24 occurs through the MAX pathway. Furthermore, differentially expressed genes between the wild-type and *max4* mutant calli, as well as GR24-treated and untreated *max4* calli, were investigated.

Chapter 6

Final conclusions and future prospects

This is a concluding discussion, linking the observations and conclusions from the previous chapters. This chapter also discusses recommendations for further studies. This thesis follows the referencing style of the *Journal of Plant Physiology*.

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Scale bars = 500µm.

List of Abbreviations

%	Percent
℃	Degree Celsius
µL	Microlitre
µM	Micromolar
2,4D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
AM	Arbuscular mycorrhizae
axr	Auxin response
BLAST	Basic local alignment sequencing tool
bp	Base pairs
cDNA	Complimentary deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
CCD	Carotenoid cleavage dioxygenase
dH_2O	Distilled water
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynuleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
EtBr	Ethidium bromide
EtOH	Ethanol
g	Grams
GA	Gibberellins
HCA	Hierarchical cluster analysis
h	Hour(s)
IAA	Indole acetic acid
IBA	Indole-3-buteric acid
IYGs	Intrinsic yield genes
JA	Jasmonic acid

KAR	Karrikin
kPa	Kilopascals
LHC	Light harvesting complex
LRR	Leucine-rich repeat
m/v	Mass per volume
M	Molar
MAX	More axillary growth
MS	Murashige and Skoog (1962)
NAA	Napthalene acetic acid
ng	Nanogram
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
PGPS	Plant growth promoting substances
PSI	Photosystem I
PSII	Photosystem II
qPCR	Quantitative real-time polymerase chain reaction
RMS	Ramosus
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Salicylic acid
Taq	*Thermus aquaticus*
λPst	Lamda DNA digested by *PstI*
µg	Microgram
µL	Microlitre
U	Units
v/v	Volume per volume
WT	Wild-type

Chapter 1
General Introduction

1.1 Background

Multicellular organisms need to optimize the utilization of available resources to suit their needs in terms of energy, biosynthetic building blocks and reserves (Meyer *et al.,* 2007). Plants, unlike animals, are auxotrophic multicellular organisms that utilize solar energy, carbon dioxide and water to make sugars and other organic compounds and thereby biomass (Demura and Ye, 2010). Biomass is a broad term that refers to material of recent biological origin that can be used as a major source of renewable energy (Bracmort and Gorte, 2012). Plant biomass has long been used by humans as raw materials for a wide range of applications including burning for energy, pulping, paper making, textiles, lumber and many other applications (Demura and Ye, 2010). The production of plant biomass depends on many factors, including the ability to capture solar energy and the conversion of this energy into vegetative tissues that can accumulate plant biomass. Accumulation of biomass in the vegetative stage of plant growth can be considered as the ultimate expression of its metabolic activities (Meyer *et al*., 2007). The metabolic status of the plant was found to be closely linked to biomass and growth (Meyer *et al*., 2007). Detailed knowledge of the interactions between biomass accumulation and metabolism may result in powerful novel tools which may accelerate and enhance the development of bio-engineered crops for plant-derived products thereof (Meyer *et al*., 2007).

1.2 Manipulation of plant biomass production

The demand for plant-derived products is increasing drastically, not only due to the growing human population; animals, research on novel biomaterials, biofuels, therapeutics and other industrial applications also rely on plants for energy (Edgerton, 2007; Gonzalez *et al*., 2007; Vercruyssen *et al*., 2011). In the upcoming years, there will be less arable land available and a higher demand for crop

production (Vercruyssen *et al*., 2011). To decrease the negative impact on the environment, increasing plant biomass production on existing agricultural land will lessen the demand for new crop acreage (Vercruyssen *et al.,* 2011). Improved agronomic practices together with traditional breeding will not support the increasing global demands and biotechnology can serve this purpose (Vercruyssen *et al.,* 2011). Marker-assisted breeding and introduction of transgenic traits for stress resistance have been shown to increase crop gain. Improving yield via the modulation of endogenous molecular pathways will be a major concern in the next generation of bio-engineered crops (Vercruyssen *et al.,* 2011). The introduction of transgenes has become widely adopted. This has been demonstrated in transgenic rice whereby three carotenoid biosynthesis genes enable the production of provitamin A, while on the other hand the new genetically engineered maize (*Zea mays*) has about eight genes which confer pesticide and insecticide resistance (Vercruyssen *et al.,* 2011).

The 1960's breakthroughs in rice and wheat production, named the Green Revolution, played a major role in increasing plant yield, and thus contributed immensely to poverty alleviation and plant productivity (Borlaug *et al*., 2007 Gonzalez *et al*., 2007). Biotechnological innovations are expected to further boost photosynthesis and produce products that are useful for humanity (Gonzalez *et al*., 2009). A unique feature of biotechnological improvement is the boosting of intrinsic yield and production of biomass with minimal demand for water, fertilizers and agrochemicals (Gonzalez *et al*., 2009). Because biomass production is very important in plant breeding and its physiological aspects, it has received considerable attention. Surprisingly, very little is known about the molecular mechanisms underpinning plant biomass accumulation (Gonzalez *et al*., 2009).
Several genes have been identified in *Arabidopsis thaliana* over the past decades which appear to be important for biomass accumulation. These are known to improve formation of larger structures (leaves or roots) when mutated or ectopically expressed and thus result in enhancement of biomass (Gonzalez *et al*., 2009; Vercruyssen *et al.,* 2011). They are commonly referred to as intrinsic yield genes (IYGs) and belong to a diversity of functional classes and operate in different pathways strongly suggesting the complexity of plant growth and biomass

(Vercruyssen *et al.,* 2011). Cross talk exists between growth controlling pathways, which create an opportunity to further improve growth by combining transgenes or making mutant alleles for genes that are involved in these pathways (Vercruyssen *et al.,* 2011). As plant hormones coordinate various plant developmental and growth processes (Wolters and Jürgens, 2009), it is no surprise that IYGs form part of the hormone biosynthetic and signalling pathways or act by influencing hormone metabolism (Vercruyssen *et al.,* 2011).

1.3 Production of plant biomass through bioactive growth substances

Plant growth regulators play significant roles in the growth and development of plants, including the regulation of meristematic activities and cell elongation, both of which are crucial for enhanced plant growth and biomass production (Gonzalez *et al*., 2009; Demura and Ye, 2010). In particular, smoke and strigolactone have been shown to be able to enhance growth in a number of plants (Cook *et al*., 1966; De Lange and Boucher, 1990; Bouwmeester *et al*., 2003; Brown *et al*., 2003; Humphrey and Beale, 2006; Chiwocha *et al.,* 2009; Light *et al.,* 2009). Smoke derived from burning plant material induces seed germination of a number of plants. About 49% of South African fynbos plants, which includes herbs, geophytes as well as trees, show a very positive germination reaction due to smoke (Light *et al*., 2009). The germination stimulating ability of smoke on both fire-dependent and independent species is well documented reported (De Lange and Boucher, 1990; Brown *et al*., 2003). Since the discovery of the growth stimulatory activity of smoke and the isolation of karrikins, which are the main bioactive compounds in smoke-water (Flematti *et al*., 2004; Van Staden *et al*., 2004), several explanations for its activity have been proposed. Karrikins can stimulate germination and promote seedling vigor at very low amounts. The action of karrikins on germination may be mediated through an interaction with some plant hormones (Brown and Van Staden, 1997). For instance, a study by Nelson *et al*. (2009) discovered the ability of smoke to enhance germination of *Arabidopsis* seed via the synthesis of gibberellic acid. The ability of smoke to interact with various hormones and to even mimic some hormonal activities has received considerable attention (Brown and Van staden, 1997, Merritt *et al*., 2005; Jain *et al*., 2008; Hayward *et al*., 2009; Soós *et al*., 2009).

Currently in South Africa, resource-poor farmers store their maize cobs over a fireplace where seeds are exposed to heat and smoke, thereby storing and protecting the grain against fungal and insect attacks (Modi, 2002; 2004). This ancient method of grain storage enhances seed germination and seedling vigour (Modi, 2002; 2004). Traditional farming systems in India have also adopted a similar method, whereby seeds of leguminous crops and maize are often kept intact by hanging over the kitchen in order to expose them to smoke (Chhetry and Belbahri, 2009). In some parts of India, fallen needles of pine trees, twigs and slashed bushes are burnt on the soil surface so as to destroy pathogens and pests. Ginger and potato crops cultivated in this manner are often very healthy (Chhetry and Belbahri, 2009). In some countries, the residue of previous crops is eliminated using fire, a pattern which is often referred to as prescribed burning. This enables farmers to speed up field operations, reduces the cost of residue management, increases biomass and thus allows control over weeds and diseases (Chen *et al*., 2005; Meland and Boubel, 1966). The advantage of burning is the removal of residues and the rapid facilitation of seed germination and seedling growth of new crops (Mandal *et al*., 2004).

Strigolactones, a group of carotenoid-derived terpenoid lactones which stem from the activity of cleavage dioxygenases from the carotenoid pathway, share some structural similarities with karrikins (Flematti *et al*., 2004; Matusova *et al*., 2005; Nelson *et al*., 2011). Strigolactones are mainly synthesized in roots and some stem parts and are often transported to the shoot apex (Humphrey and Beale, 2006; Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). These compounds are produced in a number of plant species and were first characterized as the germination stimulants of parasitic plants and later as stimulants of arbuscular mycorrhizal hyphal branching (Cook *et al*., 1972; Bouwmeester *et al*., 2003; Akiyama and Matsuzaki, 2005; Besserer *et al*., 2006). Recently, strigolactones have been identified as a new group of phytohormones, demonstrated to be regulating shoot branching inhibition and thus to control shoot architecture (Umehara *et al*., 2008; Gomez-Roldan *et al*., 2008). Additionally, a below ground role of strigolactones whereby they affect root growth in various ways has been reported. For instance, they can affect root growth via the modulation of auxin efflux (Koltai, 2010) and have been recently reported to be regulating adventitious root formation in cuttings of pea stem and hypocotyls of

Arabidopsis thaliana seedlings (Rasmussen *et al.*, 2012). Information on strigolactones may be of use in agriculture and horticulture, especially in breeding and in the development of branching regulators (Malik and Wadwani, 2009). For instance, cut flower varieties and potted plants with altered branching which may be of ornamental value may be produced; on the other hand, crops with more or less branching may benefit cultivation (Malik and Wadwani, 2009). The interaction of strigolactones in their mode of action with other phytohormones has been reported, particularly with respect to auxins and cytokinins, which play major roles in the regulation of apical dominance (Ongaro and Leyser, 2008; Muller and Leyser, 2011).

1.4 Motivation

The potential for enhancement of plant biomass production has not yet been extensively explored (Demura and Ye, 2010). Smoke-water and strigolactones have been shown to be powerful germination stimulants and they mediate a wide range of other functions for a number of plants. Research into smoke-water and strigolactones has largely focused at hormonal and mutant grafting changes to various environmental conditions and nutrient fluctuations using whole plants. The aim of this project was to further explore the great potential for promotion of plant biomass production, with a tremendous interest in the understanding of the physiological and molecular changes underlying the regulation of plant biomass, using plant cell cultures which are an alternative source to a whole plant for many physiological and biochemical studies. Subsequently, this knowledge could be applied to whole plants for the production of crops with improved biomass and higher yield.

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Chapter 2
Plant growth promotion

2.1 Introduction

Plant growth promoting substances are organic compounds that, when present in small amounts, modify physiological processes in plants (Basra, 2000; Davies, 2007). Plant growth promoting substances (PGPS) include naturally-occurring substances such as phytohormones, synthetic analogs or compounds.

The pioneering works of Went in the early 1900s left a great mark in the area of plant growth promoting substances. During those years, the term phytohormone was associated with auxins, although there were some indications of the existence of other phytohormones. In 1935, Gibberellin, which stimulated growth when applied to the roots of rice seedlings, was isolated from a fungus, *Gibberella fujikuroi* (Vivanco and Flores, 2000). Since then, research on plant growth promoting substances has advanced considerably and many more PGPS, including the well-established six major classes of phytohormones, have been identified. These comprise of auxins, cytokinins, gibberellins (GA), abscisic acid (ABA), ethylene and brassinosteroids (Ramırez-Chavez *et al*., 2004; Muller and Munne-Bosch, 2011).

Over the years, several other plant growth promoting substances that do not fall under the traditional plant hormone categories but which show an ability to influence plant growth have been discovered (Muller and Munne-Bosch, 2011). These include substances such as oligosaccharins (Fry, 1993), jasmonates (Yamane *et al*., 1980; Dathe *et al*., 1981; Ueda and Kato, 1982) salicylates (Raskin, 1992; Bennett and Wallsgrove, 1994; Gross and Parthier, 1994; Dempsey and Klessig, 1995; Hunt *et al*.,1996), polyamines (Galston and Kaur-Sawhney, 1990; Kakkar and Rai, 1993), lumichrome (Phillips *et al*.,1999), alkamides (Ramirez-Chavez, 2004), sphingolipids (Worral *et al*., 2003), humic and fulvic acid (Dobbss *et al*., 2007), plant derived smoke (De Lange and Boucher, 1990; Chiwocha *et al.,* 2009; Light *et al.,* 2009) and strigolactones (Cook *et al*., 1972; Bradow *et al*., 1988 ; 1990 ; Woo *et al*., 2001;

Snowden *et al*., 2005; Yan *et al*., 2007; Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Most of these substances are produced by fungi, bacteria and some plants and several fit the criteria to be considered phytohormones (Ramirez-Chavez, 2004; Muller and Munne-Bosch, 2011). Plant processes mediated by phytohormones include organ size control, control of fruit development, root induction, regulation of abscission, plant growth promotion and many other responses (Arteca, 1996).

2.2 Phytohormones regulate plant growth and development

Plant growth and development is made possible by the integration of a number of endogenous and environmental factors that, together with the intrinsic genetic program, determine plant architecture (Spartz and Gray, 2008; Teale *et al*., 2008; Jaillais and Chory, 2010). Phytohormones play a pivotal role in the mediation of this process (Spartz and Gray, 2008; Teale *et al*., 2008; Jaillais and Chory, 2010; Rameau, 2010). Phytohormones are small chemical compounds present in small amounts, they can function at or close to their synthesis site or can move to the target organs to elicit a response (Davies, 2007; Spartz and Gray, 2008).

Regardless of their diverse chemical structures, most of the phytohormones are derived from three main major metabolic precursors: amino acids, isoprenoid compounds and lipids. The amino acids tryptophan and methionine are precursors for Indole-3-acetic acid and ethylene respectively (Renella *et al*., 2011; Taiz and Zeiger, 2010). Five classes of phytohormones: cytokinins, gibberellins, abscisic acid, brassinosteroids and strigolactones are synthesized from the isoprenoid pathway. Jasmonic acid is produced from a lipid precursor (Taiz and Zeiger, 2010).

2.2.1 Auxin

Later in the nineteenth century, Charles Darwin and his son Francis studied the concept of plant growth involving tropisms. A phenomenon called phototropism that involves the bending of the plants toward light due to differential growth interested the Darwins (Woodward and Bartel, 2005). The Darwins' experiments involved the use of canary grass, *Phalaris canariensis,* seedlings where the youngest leaves are sheathed in coleoptiles (Eckardt, 2001). Coleoptiles serve as protecting organs and

are also very sensitive to light. Illumination with a short pulse of light, especially blue light, on one side causes them to bend toward the source of the light pulse within minutes (Taiz and Zeiger, 2006). The Darwins discovered that the tip of the coleoptiles was responsible for bending toward the light. The region on the coleoptiles that is responsible for the bending was therefore referred to as the growth zone (Taiz and Zeiger, 2006; Davies, 2007). It was observed to be some distance below the tip. It was concluded that there is a presence of a certain signal produced at the tip that travels to the growth zone. This signal resulted in the shaded side growing faster than the illuminated side (Taiz and Zeiger, 2006). Several studies were further conducted on the nature of the growth stimulus in coleoptiles. In 1926, Went demonstrated the presence of a growth-promoting substance found at the tip of *Avena sativa* coleoptiles (Delker *et al*., 2008). If the coleoptile tip was cut-off, the coleoptile's growth stopped. Several attempts in isolating the growth promoting substance failed because of the method used, until a major breakthrough by Went (Delker *et al*., 2008). Went avoided grinding and allowed the growth promoting material to pass out of the excised coleoptiles tips directly through to gelatine blocks. When this material was placed asymmetrically on top of the decapitated coleoptiles, the gelatine blocks would still be able to induce bending even in the absence of a unilateral light source (Taiz and Zeiger, 2006). Due to its ability to promote the elongation of the coleoptile sections, the name auxin was given to the plant growth promoting substance (Teale *et al*., 2006).

The most abundant naturally-occurring auxin is Indole-3-acetic-acid (Figure 2.1 A) (Normanly, 2005; Normanly, 2010). Indole-3-acetic-acid (IAA) precursors such as indoleacetaldehyde also display auxin activity. A number of other compounds such as phenylacetic acid also exhibit some auxin activity. IAA can appear in a free state or conjugated to sugars, proteins and amino acids (Davies, 2007; Delker *et al*., 2008; Normanly, 2010). Industrially-synthesised auxin analogues (Figure 2.1 B and C), such as Naphthalene-acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D), are also available (Mockaitis and Estelle, 2008). Auxin analogues exhibit similar effects to IAA and are of great economic importance (Davies, 2007; Galun, 2010).

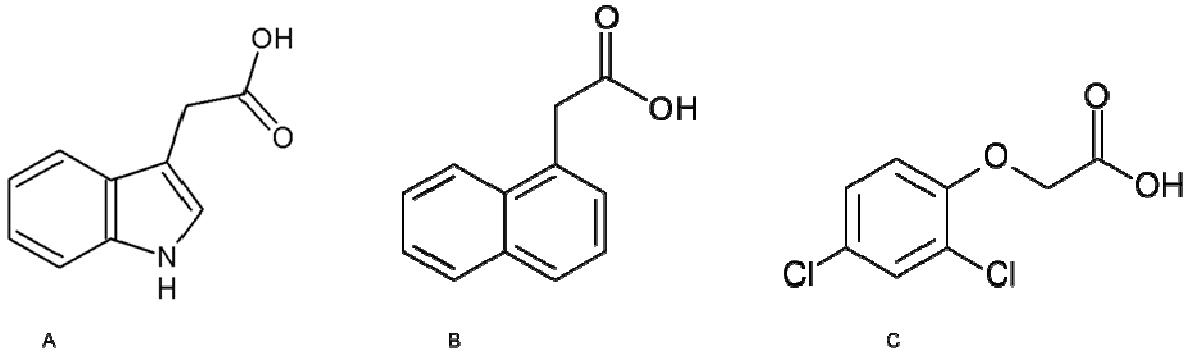

Figure 2.1 Chemical structures of Indole acetic acid (IAA) (A), Naphthalene-acetic acid (NAA) (B) and 2,4-Dichlorophenoxyacetic acid (2,4-D) (C) (Figure from Tan and Zheng, 2009)

Auxins play a very important role in various plant growth plant growth processes, such as stimulation of cell division, embryo development, root and flower development, differentiation of vascular tissue, stem elongation, tropic responses and apical dominance (Eckardt, 2001; Normanly *et al*., 2005; Mockaitis and Estelle, 2008; Woodward and Bartel, 2008).

Auxin biosynthesis is associated with rapidly dividing and growing tissues such as shoot apical meristems and young leaves (Palme and Nagy, 2008). Although root apical meristems are capable of producing auxin, especially when they mature and elongate, they still depend upon the shoot for their auxin supply (Aloni *et al*., 2003; Guo *et al.,* 2005). Seeds and young fruits also contain a vast amount of auxin, but it is still not clear whether the auxin comes from maternal tissues or is newly synthesized. A study in *Arabidopsis* young leaf primordia revealed the accumulation of auxin at the tip area (Ni *et al*., 2001). During leaf development, auxin accumulates at leaf margins and slowly shifts to the leaf base and later to the central region of the lamina. The basipetal shift in auxin production may be related to the basipetal maturation sequence of vascular differentiation and leaf development (Aloni, 2003).

There are at least three pathways involved in IAA biosynthesis. These include the tryptophan biosynthetic pathway, which begins with tryptophan decarboxylation to form tryptamine (TAM) (Lau *et al*., 2008; Woodward and Bartel, 2005; Delker *et al*., 2009). The TAM is converted to indole-3-acetaldehyde (IAAld) by a series of enzymatic reactions. The IAAld is further oxidized by specific dehydrogenases to IAA (Taiz and Zeiger, 2006). Indole-3-pyruvate (IPA) occurs in plants that do not have the TAM pathway, whilst the Indole-3-acetonitrile (IAN) pathway takes place in the Brassicaceae, Poaceae and Musacaceae families. In the bacterial pathway, indole-3-acetamide (IAM) is used as a precursor. This latter pathway occurs in various pathogenic bacteria which include *Pseudomonas savastanoi* and *Agrobacterium tumefaciens* (Taiz and Zeiger, 2006*)*.

Auxin transport is required for normal plant growth and development

Auxin transport is a very important feature for cell functioning in plants. It is essential for many plant developmental processes, such as zygote formation, differentiation and morphogenesis, responses to environmental stimuli and transport of signalling molecules like phytohormones (Lomax *et al*., 1995; Friml and Palme, 2002). Auxin is transported in two major pathways, the phloem based route and the cell to cell transport. Firstly, the fast non-directional transport via the phloem and the slower directional cell to cell route through polar transport machinery (PAT) (Friml and Palme, 2002).

Phloem-based auxin transport

This kind of route involves the transport of auxin from the main biosynthesis site towards the roots via the phloem. It is usually non-directional and occurs at a very fast rate (5–20 cm/h). The existence of phloem transport was confirmed through experiments that involved radioactively-labelled auxins (Morris and Thomas, 1978; Friml and Palme, 2002). Direct auxin analysis by Baker (2000) showed relevant amounts of free IAA within phloem exudates. In another study, the labelled auxin transported within the phloem of pea plant was later detected in the PAT stream indicating a connection between the two pathways (Cambridge and Morris, 1996).

Phloem transport is mainly associated with the transport of assimilates and inactive auxin conjugates (Nowacki and Bandurski, 1980).

Polar auxin transport

Contrary to the phloem stream, polar auxin transport (PAT) stream occurs via the polar transport machinery. It is slower (5-20 mm/h), mostly polar, runs from cell-to-cell in a basipetal manner (from the apex, the main biosynthesis site towards the root) and has a strict unidirectional character (Lomax *et al*., 1995). This kind of transport was mainly detected in the cambium and adjacent, partially differentiated, xylem elements with the use of radioactive labelled auxin (Morris and Thomas, 1978). The PAT stream is mainly responsible for the transport of free auxins (Lomax *et al*., 1995).

The auxin stream in roots moves towards the root tip acropetally. In the root tip, a certain amount of auxin is redirected backwards and moves basipetally via the root epidermis towards the elongation zone. In the shoots, PAT occurs in a lateral direction (Frilm and Palme, 2002). Assays on auxin transport showed that PAT requires energy and that it is saturable and very sensitive to inhibitors of protein synthesis. These investigations also suggested the existence of specific auxin transport proteins and eventually led to the formulation of a coherent model for auxin transport called chemiosomotic model (Rubery and Sheldrake, 1974; Raven, 1975). Chemiosmotic model describes auxin transport at the cellular level via the action of the influx and efflux carriers (Frilm and Palme, 2002; Kramer and Bennett 2006). In the acidic cell wall environment (pH 5.5), IAA appears in its protonated form (IAAH). An equilibrium shift towards the protonated lipophilic form of IAA occurs in the apoplast. This results in enhanced IAA diffusion into the cell interior and plasma membrane (Blakeslee *et al*., 2005). The cytoplasm is very basic (pH 7) and therefore cause IAAH to dissociate, consequently resulting to lipid insoluble IAA^- anions which can only exit the cell through efflux carriers (Blakeslee *et al*., 2005). Based on these observations, a specific efflux carrier was postulated and auxin flux polarity was described by its asymmetric distribution in cells. Additionally, the existence of specific auxin influx transporters was hypothesized. Years later, the saturable auxin influx, working as an $IAA^-/2H^+$ co-transporter was demonstrated (Goldsmith, 1977).

The auxin efflux and influx pathways can be distinguished by use of specific efflux inhibitors (Katekar and Geissler, 1977). The auxin influx carriers can be circumvented by the lipophilic movements, whereas the efflux carriers cannot. Thus, the cellular regulation of the efflux makes a larger contribution to the polarity of auxin transport than the regulation of auxin influx (Blakeslee *et al*., 2005).

Auxin influx

The absolute process and contribution of each of the different facilitators of auxin transport is not yet clear (Kramer and Bennet, 2006; Peer *et al*., 2011). Nonetheless, a combination of different transmembrane proteins that facilitate auxin influx and efflux (Figure 2.2) have been identified in *Arabidopsis* (Kramer and Bennet, 2006).

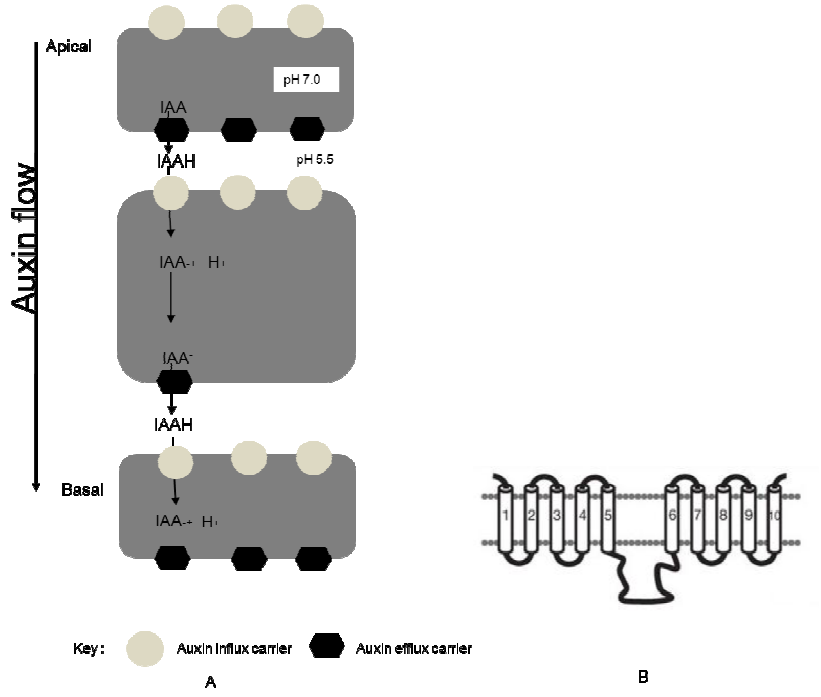

Figure 2.2 A simplified scheme showing the direction and flow of auxin transport relative to the cell (A). Auxin enters the cell by diffusion or active transport via the influx facilitator (AUX1) in the protonated form (IAAH). In the cell, IAA dissociates (IAA-) and can only exit the cell via the efflux facilitators (PIN), redrawn from Estelle, 1998). An illustration of the structure and localization of PIN1 proteins (Mravec *et al*., 2009) (B).

Auxins are highly concentrated in apical tissues, and thus the lipophilic diffusion of IAA is enhanced by a gradient-driven H^+ symport activity which is associated with the *Arabidopsis* AUX/LAX family of amino-acid permease-like proteins (Blakeslee *et al*., 2005, Zazimalova *et al*., 2010; Peer *et al*., 2011). The AUX1 protein plays a role both in root-basipetal auxin transport and in acropetal transport. Consistent with this, AUX1 shows a basal plasma membrane localization in root protophloem cells, where it functions in conjuction with apically localized efflux carriers (Blakeslee *et al*., 2005). A study in *aux1* mutants revealed decreased IAA transport in young leaf primordia and roots, reduced root gravitropism as well as resistance to inhibitory concentrations of IAA and 2,4-D. These mutants are, however, not resistant to NAA which is more lipophilic and can diffuse faster than IAA. Treatment with 1-NAA and not with 2,4-D, which is usually taken up at a similar rate to that of IAA, restores the gravitropic growth in *aux1* mutants (Blakeslee *et al*., 2005). The growth phenotypic characteristics of the *aux1* mutant are phenocopied by treatment with auxin influx inhibitors which do not affect polar auxin efflux or sensitivity to 1-NAA (Blakeslee *et al*., 2005). The AUX1 protein plays an important role both in root basipetal and acropetal auxin transport. Consistent with this, AUX1 demonstrates basal plasma membrane localization in root protophloem cells, where it functions in conjunction with apically localized efflux facilitators (Blakeslee *et al*., 2007).

Auxin efflux

Polarly-localized efflux complexes that are characterized by the PINFORMED (PIN) (Vernoux *et al*., 2001) family of proteins and multiple drug resistance/P-glycoprotein (MDR/PGPS) proteins facilitate the transport of auxin out of the cell (Blakeslee *et al*., 2005). The MDR/PGPS proteins are located in a non-polar manner in the plasma membrane of the auxin transporting cells and assist in auxin efflux through the stabilization of auxin efflux complexes. About eight members (PIN1-PIN8) of the family of the PIN proteins have been identified. They are variously expressed with growth phenotypes that show consistency in the loss of directional auxin transport in the corresponding tissue (Blakeslee *et al*., 2005). The mechanism of the facilitation of auxin transport by PIN proteins is also not clear (Blakeslee, 2005).

The PIN1 protein has a transmembrane helical structure (Vernoux *et al*., 2001) with a hydrophilic loop at the centre and is primarily localized in the xylem parenchyma (Fig 2.2B). This protein is necessary for basipetal and acropetal IAA transport in shoot and root tissues respectively. The *pin1* mutants have pinformed inflorescences, reduced basipetal auxin transport, inflorescence axes and defective vascular tissue development (Blakeslee *et al*., 2005).

The second auxin efflux protein (PIN2) is apically localized at the root cortical cells and also has a basal localization at the root epidermal tissues (Blakeslee *et al*., 2005; 2007). It plays a role in the redistribution of the auxin that is associated with root gravitropism, a process whereby a plant grows in response to gravity. The *pin2* mutants exhibit agravitropic growth phenotypes as well as reduced basipetal auxin transport in roots (Blakeslee *et al*., 2005).

Next is PIN3, a protein found at the basal region of the starch sheath cells and in pericycle cells of the root elongation zone. It functions in the lateral redistribution of auxin associated with phototropic and gravitropic growth (Blakeslee *et al*., 2005; 2007). The *pin3* mutants have decreased growth, lessened gravitropic and phototropic responses and reduced apical hook formation in etiolated seedlings (Taiz and Zeiger, 2006).

The accumulation of PIN4 protein is significant in the cells within and around the quiescent centre and can also be detected in procambial cells (Blakeslee *et al*., 2005). The PIN7 protein, like PIN3, appears at the columella cells (Blakeslee *et al*., 2005). It forms and maintains apical-basal auxin gradients that are necessary for embryonic polarity establishment. It also plays a role in root acropetal transport (Blakeslee *et a*l., 2005).

Finally, the remaining efflux proteins (PIN5, PIN6 and PIN8) have a similar trend of appearance and as a result they have been grouped together. They are found in the endoplasmic reticulum and play a role in auxin homeostasis (Mravec, 2009).

2.2.2 Cytokinins

Cytokinins are a group of structurally diverse derivatives of N^6-substituted-purines which are known for their cell division-inducing ability (Eckardt, 2003). Cytokinins (CK) were discovered more than 50 years ago, initially focussing on kinetin as a synthetic compound derived from autoclaved herring sperm DNA (Sakasibara, 2006; Davies, 2007). It was later discovered that CK are naturally occurring phytohormones that are necessary for plant growth and development (Eckardt, 2003). Several experiments by Skoog and Miller (1957) suggested the importance of the ratio of cytokinin to auxin in plant morphogenesis, with cytokinins having a greater influence on the formation of shoots and roots and their relative growth in tissue culture (Eckardt, 2003). Cytokinins influence various plant developmental processes, including the delay of senescence, nutrient mobilization, apical dominance, formation of shoot meristems, seed germination and increased crop yield (Eckardt, 2003; Werner *et al*., 2003; Sakasibara, 2006). These compounds are also implicated in light-regulated processes such as chloroplast differentiation and de-etiolation (Werner *et al*., 2003) and they usually occur in actively-dividing tissues such as developing shoots, fruits and roots of the plant (Aloni *et al.,* 2006).

Chemically, naturally-occurring cytokinins are derivatives of N^6-substituted purines (Werner, 2001). These include isopentenyladenine (iP), zeatin (Z), and dihydrozeatin (DZ), which are predominant in higher plants (Werner, 2001; Sakasibara, 2006). The strong cell division stimulating ability of cytokinins led to the identification of many compounds with cytokinin activity, such as diphenylurea and a wide number of cytokinins with aromatic side chains (Werner *et al*., 2001; Amasino, 2005; Sakasibara, 2006). The main biologically active cytokinins are the free bases, together with their ribosides (iPR, ZR, DZR) (Werner, 2001). Transportation of cytokinins is made possible by glycosidic conjugates, which are also vital for protection from degradation as well as irreversible and reversible inactivation (Werner, 2001).

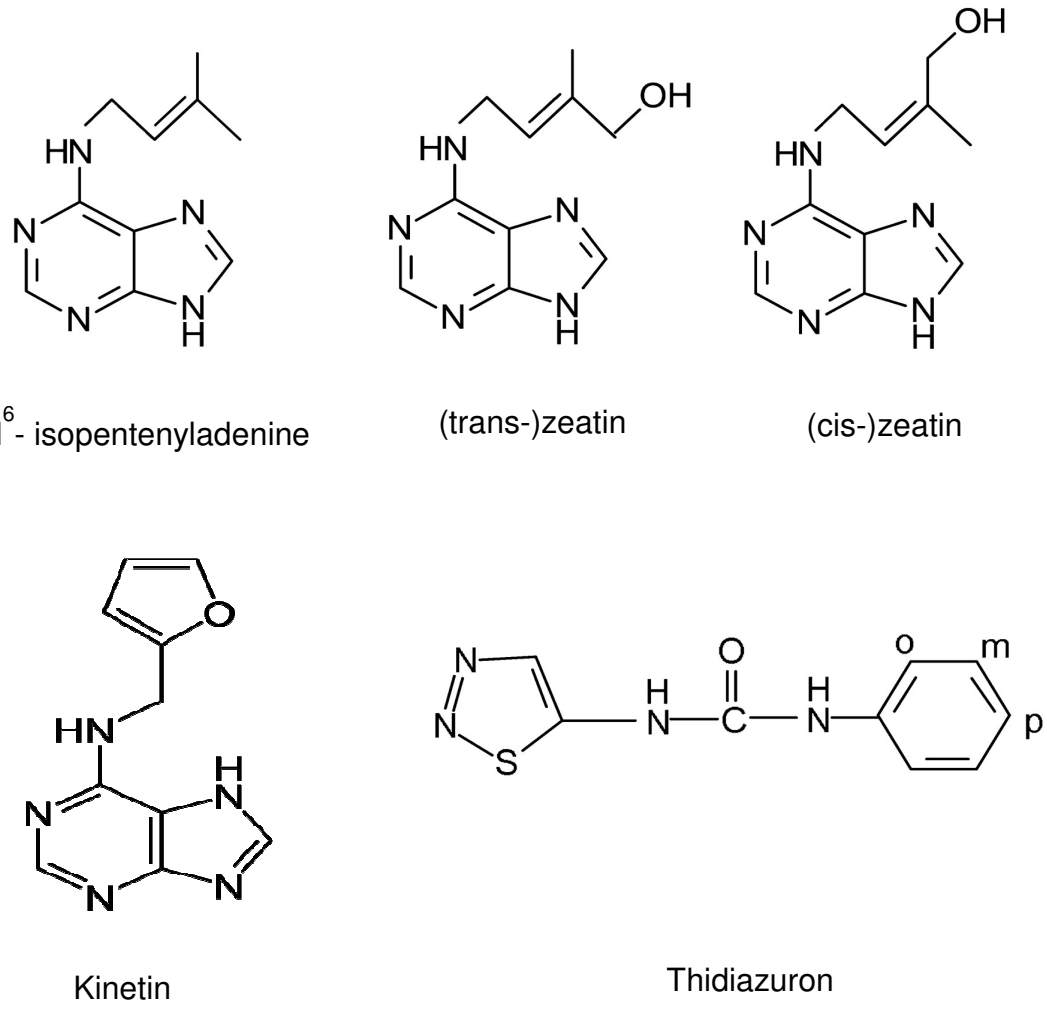

Figure 2.3 Structures of naturally occurring (zeatin) and synthetic (benzyladenine) cytokinins (www.accessscience.com).

2.2.3 Strigolactones: A newly discovered plant hormone

Strigolactones are a group of carotenoid-derived compounds found in the root exudates of a number of plants. Strigolactones were originally described as seed germination stimulants for the seeds of the parasitic weeds such as *Striga* and *Orobanche* (Cook *et al*., 1966; Bouwmeester *et al*., 2003; Humphrey and Beale, 2006). This group of compounds was later shown to also be the chemical signal for arbuscular mycorrhizal (AM) fungal symbiosis with plants (Akiyama *et al*., 2005;

Besserer *et al*., 2006). Additionally, strigolactones, or their biosynthetic precursors, were recently identified as a new class of phytohormones that regulates shoot branching inhibition (Gomez-Roldan *et al.,* 2008*;* Umehara *et al*., 2008, Dun *et al*., 2009; Xie *et al*., 2010). Recently, strigolactones have been highlighted for their new role in the regulation of root development, in Arabidopsis and pea plants (Ruyter-Spira *et al*., 2011, Rasmussen *et al*., 2012). They affect root-hair elongation and alter root architecture. Moreover, they are mediators of plant responses to the ever-changing environment (Koltai, 2011; Ruyter-Spira *et al*., 2011).

Naturally-occurring strigolactones include strigol, orobanchol and sorgolactone. Synthetic analogues of strigolactones, such as GR24, have also been produced (Johnson *et al*., 1976; Mangnus *et al*., 1992; Mangnus and Zwanenburg, 1992; Humphrey and Beale, 2006). All natural strigolactones have a common C_{19} structure with a tricyclic lactone (A, B and C rings) as a basic unit. This usually connects to a butyrolactone D-ring via an enol ether bridge. The butyrolactone D-ring is essential for the biological activity of all strigolactones (Humphrey and Beale, 2006).

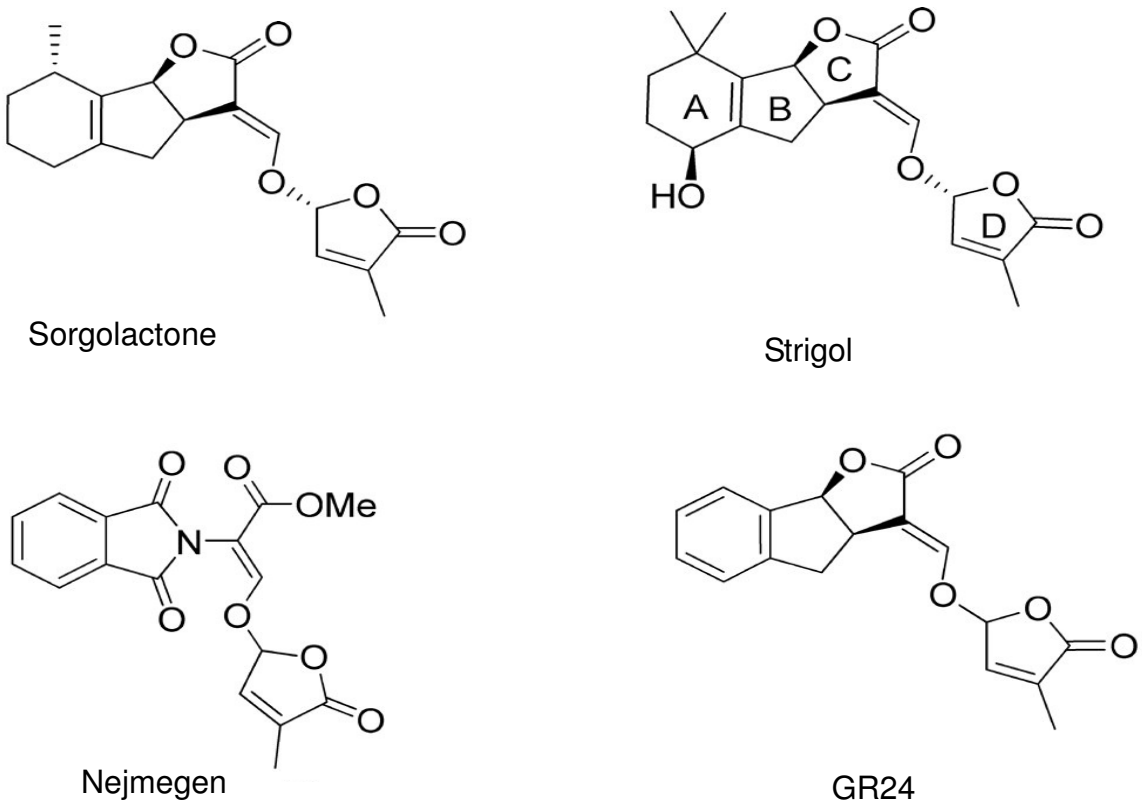

Figure 2.4 Structures of naturally-occurring strigolactones and the synthetic strigolactone analogues GR24 and Nijmegen-1 (Zwanenburg *et al.,* 2009; Mwakaboko and Zwanenburg, 2011).

Shoot branching

The plant shoot system results from the primary shoot apical meristem (SAM), which arises during embryogenesis. The primary SAM consists of many undifferentiated cells that form the main axis of the plant body (Shimizu-Sato, 2009; Ongaro and Leyser, 2008; Xie *et al*., 2010). Plant architecture is further controlled by shoot branching, which arises as a result of the activity of the additional meristems. Shoot branching controls the aerial plant architecture of the above-ground shoots (Ongaro and Leyser, 2008). Shoot branching starts with the formation of axillary buds in the leaf axils. The axillary buds formed are often dormant until they reactivate to form a branch (Xie *et al*., 2010; Ongaro and Leyser, 2008). The flexibility of the axillary meristem allows the plants to be able to modify the behaviour of the axillary buds in response to the prevailing environmental conditions (Ongaro and Leyser, 2008; Xie *et al*., 2010). For instance nutrient availability, quality and amount of light are able to

modify axillary bud activity. These environmental cues are more likely to be facilitated by the action of phytohormones, especially strigolactones, auxins and cytokinins. However, shoot branching does not only depend on environmental cues, it is also genetically controlled (Shimizu-Sato and Mori, 2001).

Shoot branching is regulated via the MAX pathway

Regulation of shoot branching is very important for plant survival and adaptation in response to the ever-changing environmental factors. Shoot branching regulation occurs via apical domance, whereby the outgrowth of lateral buds is inhibited by the shoot apex (Fergusson and Beveridge, 2009). Previous studies in Arabidopsis, pea and petunia plants suggested the involvement of a mobile signal of an unidentified compound in shoot branching inhibition, using increased branching mutants (Gomez-Roldan *et al*., 2008; Ongaro and Leyser, 2008; Umehara *et al*., 2008). The mobile signal was later identified as strigolactone (Gomez-Roldan *et al*., 2008, Umehara *et al*., 2008). The mutants studied included *more axillary growth* (*max*), *ramosus* (*rms*) and *decreased apical dominance* (*dad*) in Arabidopsis, pea and petunia plants respectively (Gomez-Roldan *et al*., 2008; Ongaro and Leyser, 2008; Umehara *et al*., 2008). Loss of function at any of these loci causes increased branching (Ongaro and Leyser, 2008). Double mutant analysis and reciprocal grafting experiments and cloning revealed that this hormone signal is a carotenoid derivative that moves acropetally and inhibits lateral bud outgrowth (Umehara *et al*., 2008). It was proposed that genes of these mutants are involved in the same pathway (Table 2.1) (Ongaro and Leyser, 2008, Beveridge *et al.,* 2009; Hayward, 2009).The strigolactone biosynthesis mutants are further discussed below.

Table 2.1 Genes identified in the strigolactone signalling pathway in four species. References: (1- Waters *et al*., 2012b; 2 - Booker *et al*., 2005; 3 - Sorefan *et al.,* 2003; 4 - Stirnberg *et al*., 2002; 5 - Waters *et al*., 2012a; 6 - Stinberg *et al*., 2002; 7- Johnson *et al*., 2006 ; 8 - Stinberg *et al*., 2002 ; 9 - Johnson *et al*., 2006 ; 10 - Lin *et al*., 2009; Liu *et al*., 2009;11 - Zou *et al*., 2006; 12 - Arite *et al*., 2007; 13 - Arite *et al*., 2009; Gao *et al*., 2009; 14 - Ishikawa *et al*., 2005; Sorefan *et al*., 2003 ; 15 - Simons *et al*., 2007, 16 - Snowden *et al*., 2005; 17 - Drummond *et al*., 2012).

	Strigolactone biosynthesis			**Signalling**		
Species	**Fe-binding protein**	**CCD7**	**CCD8**	**Cytochrome p450**	**α/β- hydrolase**	**F-Box**
Arabidopsis	*AtD27*[1]	*MAX3*[2]	*MAX4*[3]	*MAX1*[4]	*AtD14*[5]	*MAX2*[6]
Pea	-	*RMS5*[7]	*RMS1*[8]	-	-	*RMS4*[9]
Rice	D27[10]	*D17*[11]	*D10*[12]	-	D14[13]	D3[14]
Petunia	-	*DAD3*[15]	*DAD1*[16]	*PhMAX1*[17] (Not known as mutants but role described)	-	*PhMAX2a* and *PHMAX2b*[17](Not known as mutants but role described)

DWARF27

DWARF 27 (D27) is a newly identified component of the MAX pathway. It was identified from analysis of a number of rice mutants which exhibited reduced stature with more tillers compared to the wild-type (Ishikawa *et al*., 2005). Like other strigolactone mutants in rice, the *d27* mutant exhibits a highly tillered and dwarf growth habit, high level of auxin and polar transport in the shoot (Ishikawa *et al*., 2005; Arite *et al*., 2007; Lin *et al*., 2009). The D27 protein contains an iron-binding polypeptide and participates in strigolactone biosynthesis (Lin *et al*., 2009). As with other genes involved in strigolactone biosynthesis (*MAX3, D17* and *MAX4, D10*), *D27* is localized in the plastid and exhibits similar expression patterns as the D17 and D10 (Booker *et al*., 2004; Auldridge *et al*., 2006; Arite *et al*., 2007; Lin *et al*., 2009). The location and the Iron content of *D27* led to a hypothesis that it catalyses a redox reaction required for biosynthesis of strigolactone either before or after the action of *MAX3* (*D17*) and *MAX4* (*D10*) (Beveridge and Kyozuka, 2010). Recently, this hypothesis has been confirmed by the works of Alder *et al*. (2012), where D27 was identified as a β-carotene isomerise which converts all-trans-β-carotene into 9-cis-β-carotene, the CCD 7 substrate required for the next step in the pathway.

MAX3 (CCD7) and MAX4 (CCD8)

In the biosynthesis pathway of strigolactone, *MAX3*, *RMS5* and *D17/HTD1* encode plastid-targeted carotenoid cleavage dioxygenase 7 (CCD7) whilst *MAX4*, *RMS1*, D10 and *DAD1* encode CCD8 (Figure 2.5), another subclass of CCDs (Gomez-Roldan *et al.,* 2008; Umehara *et al*., 2008; Dun *et al*., 2009). The CCD proteins are nonheme iron enzymes that oxidatively cleave C-C double bonds in carotenoids and thereby produce apocarotenoids (Schwartz *et al*., 2001; Bouvier *et al*., 2003). Nine CCDs have been identified in Arabidopsis, including the 9-cis-epoxy-dioxygenase (NCEDs) class, which play a role during the production of ABA (Tan *et al*., 1997; Burbidge *et al*., 1999; Chernys and Zeevaart, 2000; Iuchi *et al*., 2001; Qin and Zeevaart, 2002).

The CCD7 and CCD8 orthologues were known to be involved in the sequential carotenoid cleavage reactions, but it was not until more recently that their substrates

and functions have been elaborated (Umehara *et al*., 2008; Alder *et al*., 2012). The two CCDs are required for the production of strigolactone upstream of MAX1. After the conversion of the all-trans-β-carotene into 9-cis- β-carotene by *D27,* CCD7 cleaves the 9-cis-β-carotene at the C9-C10 position into 9-cis-β-apo-carotenal that is further converted to a novel compound, carlactone, by CCD8. Carlactone already contains the biologically-active butyrolactone D-ring and the enol-ether bridge of strigolactones (Alder *et al*., 2012 and see figure 2.5). The final steps to the production of strigolactones are mediated by MAX1 and may include cyclisation to form the B and C rings (Alder *et al*., 2012).

MAX1

The cytochrome P450 enzyme, *MAX1* (Figure 2.5) is presumed to be involved in a later biosynthetic step of the strigolactone formation (Umehara *et al*., 2008; Gomez-Roldan *et al.,* 2008; Dun *et al*., 2009). The cytochrome P450 enzyme family occurs in almost all living organisms, including viruses, where it catalyses a vast number of redox reactions (Hannemann *et al*., 2007; Nelson *et al*., 2011). These reactions involve the movement of electrons bound through a conserved cysteine group via a haem cofactor. *MAX1* (Figure 2.5) is presumed to be involved in a later biosynthetic step of the strigolactone formation (Umehara *et al*., 2008; Gomez-Roldan *et al.,* 2008; Dun *et al*., 2009). Although MAX1 may catalyse hydroxylation reactions downstream of carlactone or the first active strigolactone compound, the details of the molecular events of its activity are still not yet clear.

MAX2

In contrast to the biosynthesis mutants, the branching pattern exhibited by *max2*, *rms4* and *d3* mutants could not be converted to that of the wild-type by grafting onto a wild-type root stock, indicating their insensitivity to strigolactones (Gomez-Roldan *et al.,* 2008; Umehara *et al*., 2008; Dun *et al*., 2009). Consistent with this idea, MAX2, RMS4, and D3 are orthologous members of the F-box family of proteins and they probably confer response to strigolactones (Beveridge *et al*., 1996; Stirnberg *et al*., 2002; Ishikawa *et al*., 2005; Stirnberg *et al*., 2007; Gomez-Roldan *et al*., 2008;

Umehara *et al*., 2008). The F-box proteins function as the substrate-recruiting subunit of the Skp/Cullin/F-box (SCF) E3 ligase complexes which are involved in ubiquitin-mediated protein degradation (Lechner *et al*., 2006; Santner and Estelle, 2010).

DWARF14

DWARF14 (D14) is a member of the α/β-fold hydroxylase superfamily of proteins. The α/β hydrolase fold is common to a number of hydrolytic enzymes of differing phylogenetic origin and catalytic function (Ollis *et al*., 1992). Proteins of the hydrolase superfamily have no obvious sequence similarities but show structural similarities with three conserved amino acid, a nucleophilic residue, acidic residue and a histidine at the centre (Nardini and Dijkstra, 1999; Ollis *et al*., 1992). They have also diverged from a common ancestor, thus preserving the arrangement of the catalytic residues. Most members of the superfamily are hydrolytic enzymes with various degrees of substrate specificities together with some proteins with no recognized catalytic functions (Nardini and Dijkstra, 1999; Ollis *et al*., 1992). Many α/β-fold hydrolase proteins play a role in metabolism and phytohormone signalling (Ueguchi-Tanaka *et al*., 2005, Kumar and Klessig, 2003; Forouhar *et al*., 2005; Arite *et al*., 2009)

The *d14* rice mutants (allelic to *d88* and *htd2*) are dwarfed and exhibit increased shoot branching phenotype that, like the previously characterized strigolactone deficient and insensitive mutants, is not rescued by exogenous application of strigolactones (Arite *et al*., 2009). Analysis of *d14* and the *d10*/*d14* double mutants suggested the involvement of D14 in the MAX/RMS/D pathway (Arite *et al*., 2009). Recently, the Arabidopsis orthologue, *AtD14* has been shown to be necessary for strigolactone responses in seedling and adult plants (Waters *et al*., 2012). As a member of the α/β superfamily, D14 has relatives both with receptor functions in plants, for example the gibberellin receptor GID1, or with biosynthetic functions (Ueguchi-Tanaka *et al*., 2005). The latter includes the salicylic acid-binding protein 2, that is essential for the production of salicylic acid (Forouhar *et al*., 2005), and the bacterial protein AidH that hydrolyses the butyrolactone ring of bacterial quorum-

sensing signal molecules N-acylhomoserine-lactones (Mei *et al*., 2010). The latter proteins share a lactone group with strigolactones (Tsuchiya and McCourt, 2012). It is thus not clear whether D14 is the last member of the biosynthetic pathway, forms part of the receptor complex or performs a step in signal transduction (Waters *et al*., 2012)

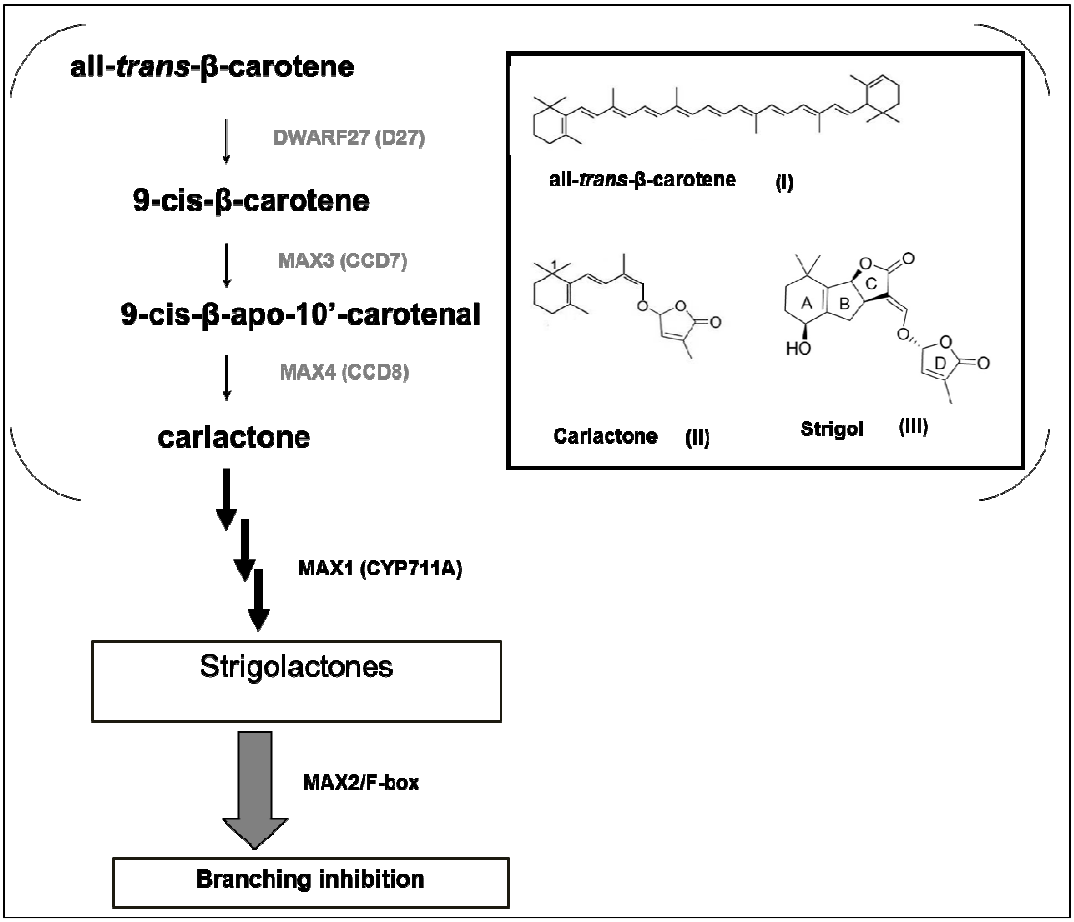

Figure 2.5 The strigolactone - dependent shoot branching inhibition pathway in Arabidopsis. D27, MAX3, MAX4 participate in strigolactone synthesis. The D27 enzyme catalyses the conversion of all-*trans*-β-carotene into 9-cis-β-carotene, that is cleaved further by CCD7 into 9-cis-β-apo-10'-carotenal, which is converted to carlactone by CCD8. Carlactone is the precursor of strigolactone and already contains the enol-ether bridge and the D-ring of strigolactones. MAX1 acts downstream of the CCDs and contributes to the conversion of carlactone into strigolactone. MAX2 plays a role in perception, downstream of strigolactone synthesis. The insert shows the structures of all-trans-β-carotene (I), Carlactone (II) and strigolactone (iii) (Modified from Umehara *et al*., 2010; Alder *et al*., 2012).

2.4 Plant derived smoke promotes plant growth

In 1990, it was discovered that smoke derived from burning plant material induces seed germination of a number of plants (De Lange and Boucher, 1990). The main germination-inducing compound in smoke was identified as the butenolide 3-methyl-2*H*-Furo [2,3-*c*]pyran-2-one (Flematti *et al*., 2004; Van Staden *et al*., 2004), which is currently referred to as karrikinolide (KAR_1). Five compounds analogous to KAR_1 have since been discovered in smoke. Together they form a family of compounds called karrikins (Chiwocha *at al*., 2009). Karrikins can stimulate germination and promote seedling vigor of a number of plants at very low levels (Light *et al*., 2009). Field trials using the parent molecule, KAR_1, demonstrated its effect at less than 5g per hectare, an indication of commercially-viable application rates (Stevens *et al*., 2007). Fractions of smoke-water containing KAR_1 have been reported to enhance seedling vigor of a number of plants, suggesting KAR_1 as a potential tool in agriculture and crop improvement strategies (Jain *et al*., 2006; Jain and Van Staden, 2006; Kulkarni *et al*., 2006; Van Staden *et al*., 2006; Daws *et al*., 2007). A widespread germination response to KAR_1 has been shown amongst angiosperms, since its discovery (Flematti *et al*., 2004; van Staden *et al*., 2004, 2006; Merritt *et al*., 2006; Daws *et al*., 2007; Stevens *et al*., 2007). Therefore, karrikins may be considered a new class of plant growth regulators with broad impact (Nelson *et al*., 2009).

Plant-derived smoke has been shown to stimulate the germination of more than 1200 species located in various regions around the world, including South Africa, Australia, Europe and California (De Lange *et al*., 1990; Baldwin *et al*., 1994; Dixon *et al*., 1995; Brown *et al*., 1997; Dixon *et al*., 2009). Intriguingly, smoke is not only effective on plants from fire-prone habitats as plants from non-fire prone areas also responded positively to smoke treatment (Drewes *et al*., 1995; Pierce *et al*., 1995; Daws *et al*., 2007). Much evidence has been formulated on the growth stimulatory effect of smoke-water or smoke-derived compounds both in crop and weed species (Adkins *et al*., 2001). During these investigations, smoke/smoke-derived compounds have been shown to be a significant germination stimulator and a convenient treatment for improving seedling vigour. For instance, a number of crop plants have

responded positively to smoke-water/smoke-derived compounds; including celery, tomato, lettuce, okra, rice, bean and maize (Van Staden *et al*., 2006). The broad functions of smoke-water in the regulation of plant growth, as well as its emerging application as an ecological and land restoration tool, could provide benefits for horticulture and agriculture (Brown and Van Staden, 1997; Light *et al*., 2009). The application of smoke-compounds to plants might increase crop productivity and may be a cost effective and affordable method for resource poor farmers, especially in developing countries. However, the effects of KAR_1 and other smoke-derived substances need to be further investigated in order to really understand their full potential as a tool for improving agricultural yields and commercial application (Kulkarni *et al*., 2011).

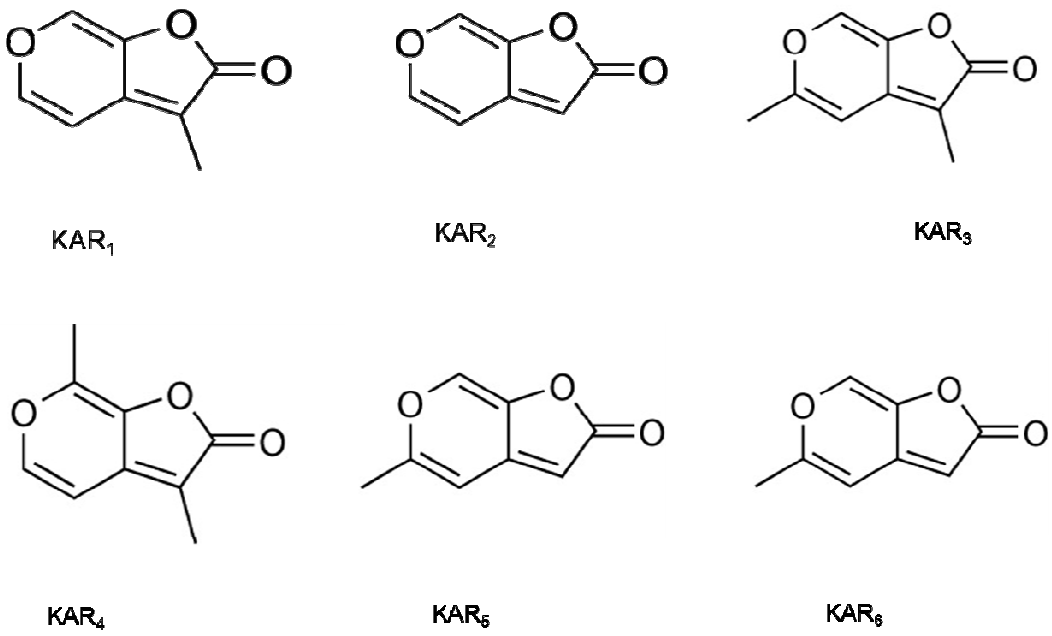

Figure 2.6 Chemical structures of karrikin (KAR_1 – KAR_6) plant growth regulators (Chiwocha *et al*., 2009; Nelson *et al*., 2012).

Relationship between karrikins and strigolactones

Strigolactone perception is linked to the D-ring structure and the enol-ether bridge which connects the D-ring to the rest of the compound. Evidence for this has been shown by testing a number of other synthetic strigolactones which do not have either or both of these synthetic characteristics (Zwanenburg *et al.,* 2009). Karrikin

compounds have a furanone moiety that is similar to the D-ring of strigolactones. Previously it had been shown theoretically that the putative reaction between strigolactones and the unknown receptor could potentially also occur in response to KAR_1 (Zwanenburg *et al*., 2009). Strigolactones and karrikins have both common and distinct responses in plants (Nelson *et al*., 2011). Their common effects include stimulation of seed germination, seedling photomorphogenesis, and expression of a small set of genes during these developmental processes (Nelson *et al*., 2011). Karrikins are also able to repress the transcripts of MAX4 and IAA, which exhibit negative feedback regulation through strigolactones (Nelson *et al*., 2011).

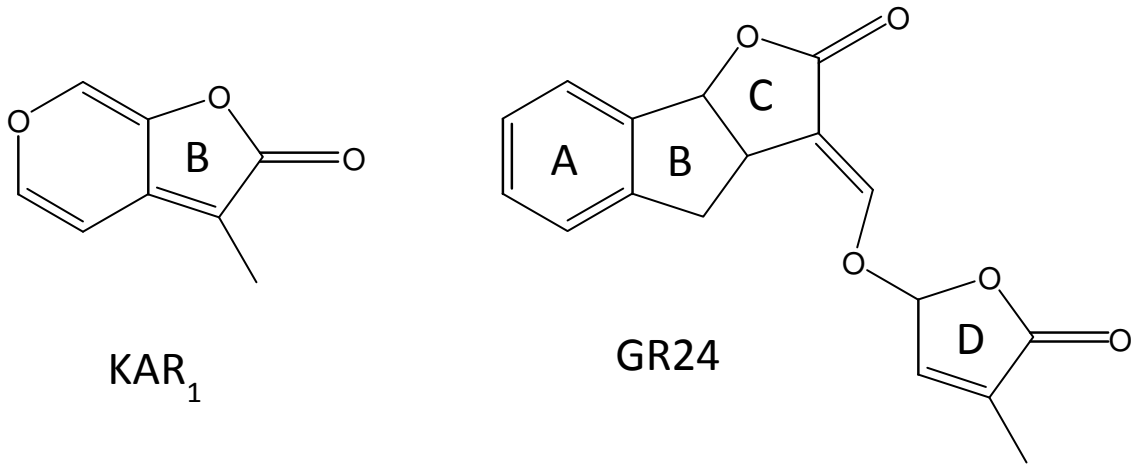

Figure 2.7 The B-ring of KAR_1 is identical in structure to the D-ring in strigolactone (adapted from Zwanenburg *et al*., 2009).

Similar responses to strigolactones and karrikins are not due to the butenolide moiety only. There were two hypotheses that were proposed to link the two (Nelson *et al.,* 2011). The first hypothesis suggests that both of these substances have different signaling mechanisms and receptors completely. Another hypothesis suggested a common signal transduction mechanism that might be shared by these compounds (Nelson *et al*., 2011). Recently, genetic screen studies in *Arabidopsis thaliana* for karrikin-insensitive mutants have revealed the importance of the F-box *MAX2* gene in karrikin signaling. Four separate genetic screens demonstrated the roles of this protein in seed germination, shoot outgrowth, leaf senescence and seedling photosensitivity (Waters *et al*., 2011). Both karrikins and strigolactones can

regulate seed germination and seedling photomorphogenesis via MAX2. However, unlike strigolactones, karrikins cannot inhibit shoot branching, indicating the ability of plants to distinguish between these signals (Nelson *et al*., 2011; Waters *et al*., 2011). However, it remains unclear how discrete responses to these two classes of compounds are achieved (Waters *et al*., 2011; 2012).

Recent discovery show that *KARRIKIN INSENSITIVE 2* (*KAI2*), an *AtD14* paralogue, is necessary for responses to karrikins. Mutants in *AtD14* and *KAI2* largely share similar phenotypes with *max2* and are also insensitive to strigolactones or karrikins. The *max2* mutants are themselves insensitive to karrikins, suggesting that both MAX2 and KAI2 are required for responses to karrikin. Mutants of both *MAX2* and *KAI2* demonstrate germination deficiencies, whilst the germination profile of *Atd14* mutants resembles that of the wild-type. Therefore, the MAX2/KAI pair is mainly required for seed germination, whereas the MAX2/D14 pathway plays important roles in aother strigolactone responses such as the regulation of shoot branching (Waters *et al*., 2012).

The KAI2 proteins belong to a different group of the α/β-fold hydrolase than AtD14 (Waters *et al*., 2012). The different members of the α/β-fold hydrolase allow the different regulation of strigolactone and karrikin signalling by MAX2 (Waters *et al*., 2012). The only characterized member of the α/β family is the RSbQ from the bacterium *Bacillus subtilis.* Brody *et al*. (2001) suggested the *RSbQ* gene to be a regulator of energy-stress responses in *Bacillus subtilis*. Amino acid analysis revealed that AtD14 and KAI2 are 38% and 39% identical to RSbQ, furthermore, they also share the hydrolase catalytic triad of Ser96, Asp219 and His247 residues (Waters *et al*., 2012).

Phylogenetic analysis revealed that land plant sequences form two sister clades of the α/β-fold hydrolases, the DWARF14 group, consisting of the rice founding member OsD14 and AtD14, and the D14-LIKE family, consisting of the rice OsDLK1 and AtKAI2 (Waters *et al*., 2012). Interestingly, the D14-LIKE clade appears to be more ancient and taxonomically diverse, encompassing byrophytes, gymnosperms

and angiosperms. It appears that the DWARF14 sequences are as a result of a gene duplication event within the line of descent of vascular plants, as this clade contains more recently divergent homologues. The tentative conclusion to draw is that the duplication of D14-LIKE resulted in the evolution of parallel pathways which can both sense molecules (Karrikins and strigolactones) whose presence predates in *planta* roles and which share structural similarities and at the same time retain an elegant efficiency by sharing components of downstream signal transduction (Waters *et al*., 2012).

2.5 The ubiquitin proteasome system is essential for phytohormone signalling

Many aspects of plant‘s life depend on regulated proteolysis to control the manufacture of new proteins and the degradation of pre-existing ones. This highly conserved mechanism controls the supply of amino acids for constructing new proteins, eliminates abnormal or redundant proteins and dismantles existing regulatory networks (Hellmann and Estelle, 2002; Vierstra, 2003). The mechanism of proteolysis is very specific, tightly regulated and is crucial for the survival of all organisms (Sullivan *et al*., 2003). It also allows plants to switch from one developmental state to another in response to new environmental signals and conditions, in order to direct growth and development to the ever-changing environment (Sullivan *et al*., 2003). There are many proteolysis mechanisms used by eukaryotes to modulate their protein levels. In all organisms, the most prominent of these is the ubiquitin/26S proteasome system (Ub/26S) (Sullivan *et al*., 2003; Santner and Estelle, 2010). Ubiquitin is a 76-amino acid protein which covalently attaches to the target protein via a terminal carboxyl extension. This process takes place through the activity of three enzyme families, which are Ub activator (E1), Ub conjugating enzyme (E2) and Ub ligase (E3) (Eckardt, 2001; Vierstra, 2003; Moon and Estelle, 2004). The ubiquitin activating-enzyme (E1) activates ubiquitin by forming a thiolester bond between itself and ubiquitin, E2 then binds to the activated E1-ubiquitin before the action of E3 which functions together with E2 to transfer the activated ubiquitin to a target protein (Eckardt, 2001; Vieratra, 2003; Moon and Estelle, 2004). The repeated activity of these enzymes generates a polyubiquitinated

protein (multiple Ub subunits) which is recognized and then degraded by the 26S proteasome complex, with concomitant release of free ubiquitin moieties for re-use (Eckardt, 2001). The overall ubiquitination process occurs in an ATP-dependent manner (Vierstra, 2003).

There are two groups of E3 ligases that have been identified in plants, these can be distinguished according to the type of interaction domain (RING /U-box domains or HECT domains) used to bind E2 enzymes and function (Santner and Estelle, 2010). In *Arabidopsis*, the most abundant E2 interaction domain is found in the Really Interesting New Gene (RING) and the closely related U-box proteins, which are collectively referred to as the RING group. RING proteins frequently interact with other proteins to forma complex called the SCF factor. The fingers consist of amino acids that bind zinc. The U-box exploits electrostatic interaction to stabilize a RING-finger like structure. The second group is called HECT E3s, and is made up of very large proteins. About seven HECTs have been identified in *Arabidopsis* (Moon *et al*., 2004). The RING/U-box serves as an Ub-E2 docking site, activating the transfer of ubiquitin to the substrate (target) lysines (Santner and Estelle, 2010).

The SCF E3 ubiquitin ligase consists of a complex of several polypeptides that also function as scaffold to bring together the activated Ub-E2 complex and the target, causing conjugation without forming an E3-Ub intermediate (Fig. 2.8). The SCF group of E3 ligases is the most abundant and thoroughly studied in plants. The SCF name is derived from three of its four subunits i.e. SKIP, Cullin (CDC53) and the F-box protein. A RING finger protein, RBX1, makes the fourth subunit (Moon *et al*., 2004; Santner and Estelle, 2010). In this complex, CUL1 serves as the backbone and binds both RBX1 at the C-terminus and the linker protein SKIP at the N-terminus. The SKIP protein in turn binds to the F-box domain of the F-box protein to complete the complex (Santner and Estelle, 2010).

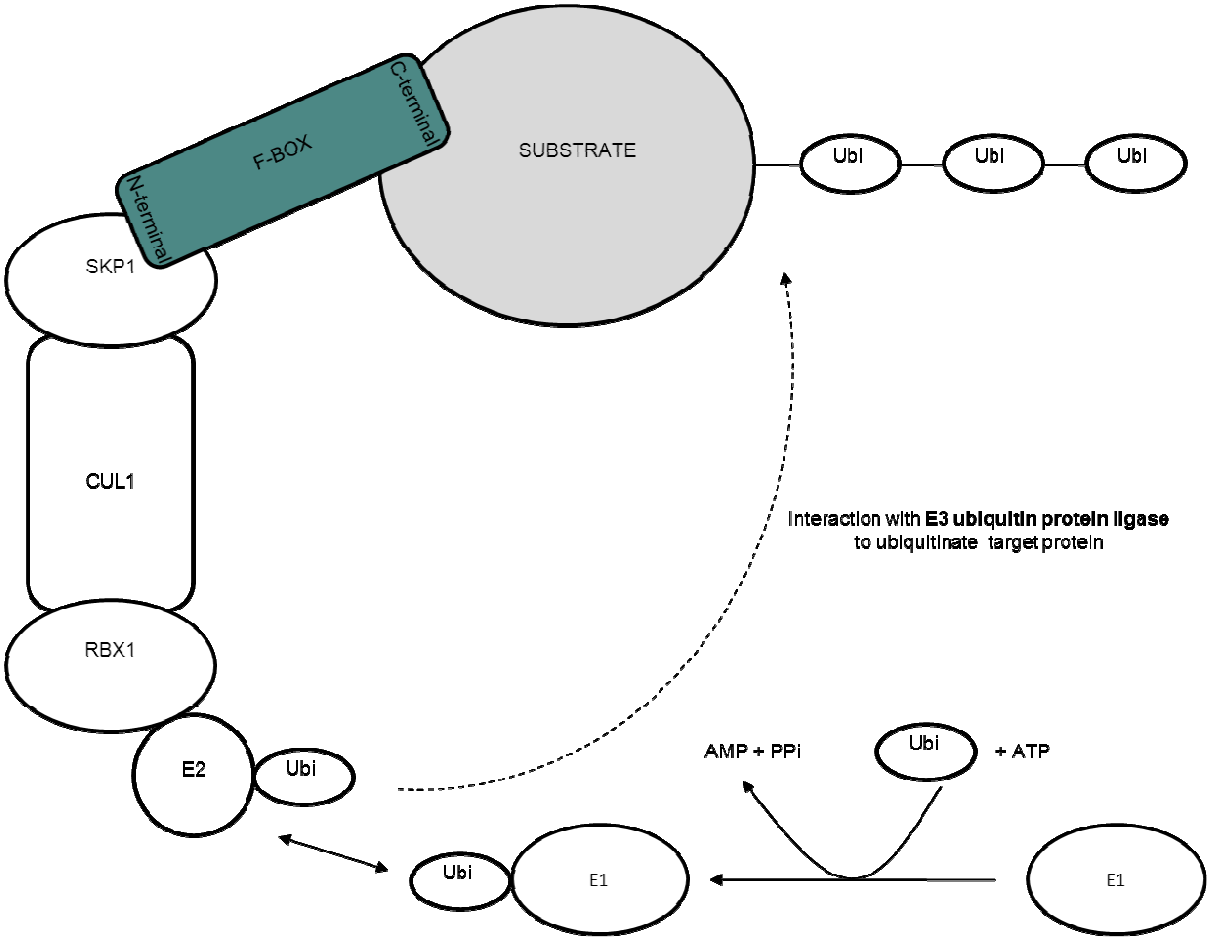

Figure 2.8 A simplified diagram of the SCF ubiquitin complex. The major components of the complex (SKP1, Cullen and F-box proteins) are illustrated. The N-terminal of the F-box protein interacts with the SKP protein to form the SCF complex. The C-terminal of the F-box protein binds to target substrate proteins which are then polyubiquitinated by the E3 ubiquitin ligase and then degraded by the 26S proteasome.

Such SCF E3 ligases are critical components in the regulation of responses to several phytohormones. For instance, the involvement of the ubiquitin-proteasome SCF complex in auxin response has been shown (Santner *et al.,* 2009). The role played by SCF complexes in auxin signalling began with the identification of the auxin receptor TIR1, an F-box protein belonging to a family of F-box proteins that includes additional auxin-signaling F-box proteins (AFBs) (Eckdart, 2001; Dharmasiri *et al*., 2005). Auxin action is mediated by binding to TIR1/AFBs, which results in the

degradation of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional repressors via the SCF^{TIR1} complex by the 26s proteasome (Liscum and Reed, 2002; Reed *et al*., 2001; Zenser *et al*., 2001; Dharmasiri *et al*., 2005; Gray, 2001). The degradation of AUX/IAA frees up the binding sites for the ARFs (AuxRE), and thus permitts the ARFs to bind to the promoter regions of auxin-responsive genes, resulting in the expression of target genes required for plant growth. The discovery that AUX/IAA proteins are targeted for degradation via the SCF^{TIR1} complex was a major breakthrough in the characterization of the auxin-signaling pathway (Santner *et al*., 2009). Recessive mutations in *TIR1* result in auxin resistance, indicating the necessity of this protein for the degradation of negative auxin response regulators (Dharmasiri *et al*., 2005).

Gibberellic acid regulates a variety of plant growth and development processes (Wang *et al*., 2009). Over the years, a number of factors that affect GA perception have been identifiedmainly through genetic screens in rice and *Arabidopsis*. In particular, GA-insensitive mutants with defects in genes encoding F-box proteins have been identified (Hirano *et al*., 2008). Responses to GA are induced by the degradation of DELLA proteins, a group of nuclear-localized transcription regulators functioning as repressors in GA signal transduction (Harberd, 2003; Wang *et al*., 2009; Santner and Estelle, 2010). Gibberellins regulate the abundance of transcription repressors by promoting the ubiquitination of DELLA proteins through the SCF-type E3 ligases (Santner and Estelle, 2010). The *Arabidopsis* F-box protein SLEEPY1 (SLY1) and rice GIBBERELLIN-INSENSITIVE DWARF2 (GID2), involved in GA signalling have been identified (Santner and Esstelle, 2010). Their respective SCF complexes, SCF^{SLY1} and SCF^{GID2}, facilitate the degradation of the DELLA proteins such as the REPRESSOR OF GA1-3 (RGA) and SLENDER RICE1 (SLR1) by the 26S proteasome (Ueguchi-Tanaka *et al*., 2005; Santner and Estelle, 2010).

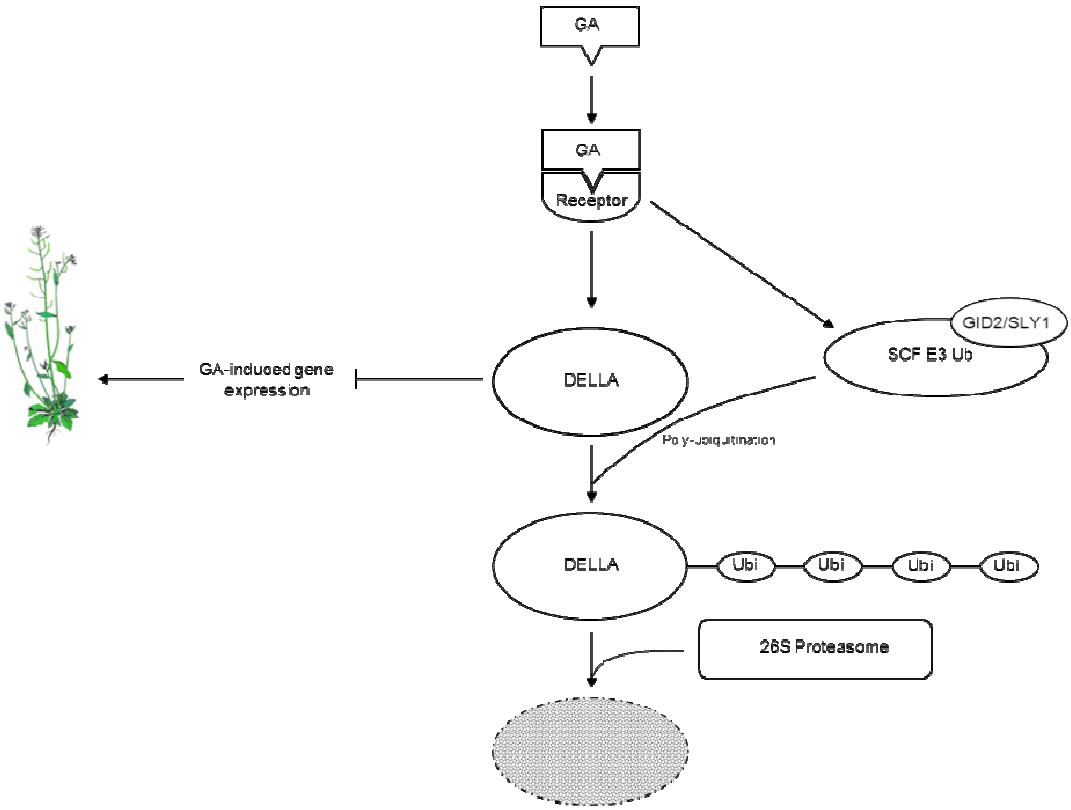

Figure 2.9 An illustration of the SCF ubiquitin ligase complex in action during GA perception. In response to a GA signal, the DELLA proteins are targeted for destruction in the 26S proteasome by polyubiquitination. Degradation of DELLA proteins releases the restraint on plant growth, (adapted from Harberd, 2003 with modification).

Arabidopsis has three nuclear-localized GID1 genes (GID1a, GID1b and GID1 c), which are implicated in GA perception (Ueguchi-Tanaka *et al*., 2005; Eckardt, 2006; Griffiths *et al*., 2006; Hirano *et al*., 2008; Voegele *et al*., 2011). The GID1 protein is a member of a hydrolase family which shares homology with the hormone sensitive lipase family (HSL) (Ueguchi-Tanaka *et al*., 2005). Strong evidence that suggests a prominent interaction between DELLA proteins and GID1 has been provided (Griffiths *et al*., 2006). Experiments demonstrated that the GA-GID1 complex promoted direct binding of the F-box proteins SLY1/GID2 in the presence of GA (Hirano *et al*., 2008). Thus, upon binding to GA, GID1 derepresses the GA response pathway through binding to DELLA proteins and thereby promoting their degradation by the $SCF^{SLY1/GID2}$ proteasome system (Griffiths *et al*., 2006). These observations

therefore suggested that DELLA proteins can better interact with the $SCF^{SLY1/GID2}$ while in a complex with GA-bound GID1 (Griffiths *et al*., 2006).

Another example where F-box proteins are implicated in response is that of MAX2, which has been proposed as a candidate for strigolactone perception. Recently, MAX2 has also been reported to be involved in mediating responses to the structurally-related karrikins (Nelson *et al*., 2011) as explained above. It is suggested that strigolactones and karrikin bind to MAX2, resulting in ubiquitination of specific target proteins by SCF^{MAX2} which are eventually degraded by the 26S proteasome (Lumba *et al.,* 2010; Santner and Estelle, 2010; Waters *et al*., 2011). It is intriguing that MAX2 is structurally similar to TIR1 and CORONATINE INSENSITIVE1 (COI1), another F-box protein that is required for response to the plant hormone jasmonic acid (Gagne *et al*., 2002, Mashiguchi *et al*., 2009). The structural similarities of MAX2 to these receptors suggest that MAX2 is a receptor component of the strigolactone receptor complex. It is also tempting to speculate that MAX2 might also act directly as a receptor like TIR1 and COI1 (Mashiguchi *et al*., 2009). However, reports by Arite *et al*. (2009) provided an alternative possibility. This occurred after the discovery of a rice gene D14/D88/HTD2, which has also been shown to encode a member of of the α/β-fold hydrolase superfamily as described above. The D14/D88/HTD2 is structurally similar to GID1, which is also a member of this family. These similarities strongly suggest a possibility that similarly to the GA system where GID1 is a receptor that binds to SLY1 when bound to GA, D14/D88/HTD2 may be a receptor of strigolactones (Ueguchi-Tanaka *et al*., 2005; Arite *et al*., 2009; Beveridge and kuyozka, 2010). These findings strongly suggest the importance of protein degradation in response to phytohormones (Santner and Estelle, 2010).

Among the 692 F-box proteins identified in *Arabidopsis*, MAX2 belongs to a subfamily of 33 members with a similar arrangement of F-box LRR domain. Many members of this subfamily play major roles in regulation of plant growth and phytohormone signalling (Waters *et al*., 2011). Findings about MAX2 could vastly contribute in various areas of agriculture. Currently in Australia, researchers are already applying smoke to restore vegetation in ex-mine areas and ex-plantation

sites in ecological management and restoration strategies (Ruthrof *et al*., 2011). With the recent findings about the role of MAX2 in karrikin perception and seed dormancy, it is anticipated that additional functions for this protein will be discovered in future. Identifying the protein targets of the SCF^{MAX2} complex is a crucial area of future research that will bring more understanding on this aspect (Waters *et al*., 2011). More light on this can potentially contribute to biotechnological strategies in attempt to directly manipulate crop plants to possess certain desired traits.

2.6 *Arabidopsis thaliana* as a model to study plant development

Plant growth and development is a very complex process (Vercruyseen *et al*., 2011). Therefore, the use of model organisms that allow optimal experimental strategies has become a common practice in plant growth studies (Scheres and Wolkenfelt, 1998). *Arabidopsis thaliana* is a small flowering plant that belongs to *Brasicaceae* (mustard) family (Meinke *et al*., 1998). It is found in Asia, Europe, North America, Australia and some parts of Africa. This species has been extensively used in plant biology as a model organism because of its characteristics (Francois *et al*., 2008). Studies using *A. thaliana* include genetics, cellular and molecular biology as well as growth and development of plants. It has a fully sequenced, small genome (115 Mb-125 Mb), with genetic and physical maps of all chromosomes available (Page and Grossniklaus, 2002). A variety of genomic resources and mutant lines are available. The small size of *A. thaliana* makes it convenient for cultivation in confined spaces and it yields a large production of seeds. Furthermore, it has a rapid life cycle of about 6 weeks from germination to mature seed (Sun *et al*., 2009). There are also efficient methods for transformation with other microorganism such as *E. coli* and *Agrobacterium tumefaciens* (Meinke *et al*., 1998).

2.7 Plant tissue culture as a scientific tool

Plant tissue culture is a collection of techniques that are used to grow or maintain plant cells, tissues or organs in a sterile environment (Smith, 2000). Small pieces called explants are used in plant tissue culture initiation. Explants can be isolated from any part of a plant. Practically, the explant is excised, surface decontaminated

and placed on a nutrient medium to start a mother culture that is multiplied by sub-culturing (Nasaru, 2000; Chawia, 2004). A suitable nutrient medium is vital to success in tissue culture (Smith, 2001). Additionally, suitable optimization of the medium for a specific organ, cell line or tissue of interest must be performed (Narasu, 2000; Smith, 2001). The medium can either be liquid with necessary growth nutrients or solidified by adding gellan gum or agar. The tissue culture growth nutrients include inorganic micro and macro elements, organic nutrients such as amino acids and vitamins, plant growth regulators (hormones), as well as a carbon source (Smith, 2000; Neuman *et al*., 2009). Usually, the nutrient medium replaces the cells, tissues and conductive elements surrounding the explants (Neuman *et al*., 2009). The application of cell and tissue cultures aims at better understanding of the physiological, biochemical, anatomical as well as molecular effects of selected cell material to specified factors under controlled conditions (Kumar and Loh, 2012). This is done in an attempt to gain more insight into certain aspects of the life of intact plants, as well as their natural environment (Kumar and Loh, 2012).

The main advantage of using tissue culture systems is that they offer easy control of chemical and physical environmental factors compared with the use of intact plants. These chemical and physical environmental factors can be kept constant at reasonable costs (Ahloowalia, 2004). The growth and development of many plant organs can be investigated whilst excluding the influence of remote material in the intact plant body. In synthetic culture media available in many formulations today, the effects of a given cell material to certain factors can be investigated (Neuman *et al.,* 2009). For instance, the influences of nutrients or plant growth promoting substances on development and metabolism associated with tissue growth can be investigated through the use of cell and tissue cultures. These were some of the approaches invented by the fathers of tissue culture in the earliest 20th century and they are still applied today (Neuman *et al.,* 2009). The cell and tissue culture field is very broad including micropropagation, callus cultures, seedling cultures, embryo, protoplasts, pollen grain culture, shoot apices, cell suspensions, anther cultures as well as organ or meristematic cultures (Hartman *et al.,* 2002; Idowu *et al.,* 2009; Neumann *et al.,* 2009).

Plant cell cultures have been utilized as model system to circumvent problems associated with the analyses of a whole-plant that consists of multiple cell types and tissues exposed to diverse signals . The majority of these cultures have proven to be valuable tools in a number of plant growth and development studies including secondary metabolites formation, carbohydrate metabolism, gene regulation, ion transport, defense responses and signal transduction pathways (Sinha *et al*., 2002; Sharathchandra *et al*., 2011). The establishment and maintainance of *Arabidopsis thaliana* cell cultures has been described (Goldy, 1989). This aspect together with the abundant information and molecular genetic tools reported for this species have increased interest in its cell cultures for its use a model for molecular and biochemical studies (Tjellstrom *et al*., 2012). *Arabidopsis thaliana* cell cultures have been used previously in various studies including cell wall protein analysis, lipid composition and metabolism (Borderies *et al*., 2003; Tjellstrom *et al*., 2012). However, *Arabidopsis* cultures have not yet been specifically tested for their suitability to study the dynamics of biomass accumulation.

2.8 The aim of the study

Smoke and strigolactones both have remarkable effects on seed germination in a number of plants. Responses to smoke water or GR24 treatment other than having a germination stimulating ability have also been reported. While research into the effects of smoke-water and strigolactones in plant growth and development has mainly focused on physiological responses using intact plants, there is not enough information on their implication in the growth of cell cultures. *Arabidopsis* cell cultures have been used in a variety of studies previously, including cell wall protein analysis, lipid composition and metabolism (Borderies *et al*., 2003; Tjellstrom *et al*., 2012). It would be interesting to test for the suitability of *Arabidopsis thaliana* callus cultures as an alternative model system to study the dynamics of plant growth, specifically biomass accumulation using smoke-water and strigolactones. Whilst exploring the potential for enhancement of plant biomass by these two plant growth promoting substances, their function in an undifferentiated state of cells would be elucidated. Furthermore, smoke-water and strigolactones share structural similarities and may trigger similar plant responses, could they affect the growth of callus in a similar

manner? Findings on this could create more knowledge and significantly make a great contribution to the study of the intricate regulatory mechanisms and signaling networks responsible for biomass accumulation.

The aims and objectives of this study were to establish *in vitro* callus cultures of *Arabidopsis thaliana* to provide a biotechnological tool and to study the effects of two plant growth promoting substances (smoke-water and strigolactones) on the growth of these cultures.

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Chapter 3
In vitro callus induction of *Arabidopsis thaliana*

3.1 Introduction

3.1.1 Callus cultures

Callus refers to a group of undifferentiated cells or non-organized tumour tissue which usually arises on wounds of injured tissues or organs (Chawla, 2002). Callus induction begins with a tiny section of plant tissue or explant that is manipulated with the use of plant growth regulators to produce calli (Chawla, 2002). Tissues with high contents of parenchyma or meristematic cells are most convenient in starting a callus culture (Neumann *et al.,* 2009), due to the fact that such tissues have a small number of cell types with high histological homogeneity compared with the entire organ (Neumann *et al.,* 2009). An equal ratio of auxins and cytokinins usually results in the desired effect, although this hormone combination varies depending on the species used (Chawla, 2002). The type of growth regulator applied and the concentration required also depends on the endogenous hormone concentration as well as the genotype of the explants used (Chawla, 2002). Callus cultures aid in the multiplication of limiting plant material, as well as in the isolation of somaclonal variants (Neumann *et al.,* 2009*)*. The earliest studies in *Arabidopsis thaliana* callus formation were done by Loewenberg in the mid-1960s. Since then, numerous studies in tissue and cell culture in both liquid and solid media have been published (Huang and Yeoman, 1983).

The aim of this chapter was to establish an efficient protocol for rapid production of *Arabidopsis thaliana* callus cultures. The proliferated explants were used to multiply calli *in vitro*, which was important to develop a stock culture for further investigations into the feasibility of enhanced biomass production using smoke-water and strigolactones.

3.2 Materials and methods

3.2.1 Preparation of explant material

Arabidopsis thaliana seeds were the experimental materials in this present study. Seeds of the *max1-1* (N9564), *max2-1* (N9567), *max2-2* (N9566), *max3-9* (N9567), *max4*-1 (N9568) homozygous mutant lines and their parental Columbia-0 wild-type line were purchased from the Nottingham Arabidopsis Stock Centre (NASC). All operations were carried out in a laminar airflow cabinet under sterile conditions. Approximately 50 seeds were placed in a microfuge tube. Surface decontamination was accomplished by washing the seeds with 1 mL of a disinfecting solution with periodic agitation for 5 minutes. The disinfecting solution contained 96% (v/v) ethanol, 10% (m/v) sodium hypochlorite, Tween 20 (two drops in 50 mL) and sterile distilled water. After three washes in sterile distilled water, the seeds were poured onto sterile filter papers in glass Petri dishes, prior to transfer onto agar medium in 90 x 10 mm diameter growth plates (Greiner Bio-One, Cellstar®) for germination. The agar medium consisted of basal Murashige and Skoog (1962) mineral (MS) salts (Highveld Manufacturing), 3% (m/v) sucrose and was solidified with 0.2% (m/v) Gelrite. The growth promoting substances used were combinations of 2 mg/L 2,4-D and 2 mg/L kinetin (growth medium C1) or 0.5 mg/L 2,4-D and 0.05 mg/L kinetin (growth medium C2). The pH was adjusted to 5.8 with KOH before addition of the Gelrite and all media were sterilized by autoclaving at 121℃, 100 kPa for 20 minutes. A maximum of 15 seeds were placed onto each plate of 6 replicates per genotype. For synchronisation purposes, the seeds were covered with foil and stratified at 4℃ for 48 hours. Seeds were then germinated in a growth room at 23±2℃ under cool fluorescent lights (Osram L 58V/740) using a 16/8 hour light/dark regime (50 µmol photons $m^{-2}s^{-1}$), prior to placing them in the dark for 5-7 days to etiolate.

3.2.2 Callus production

When the seeds had germinated and the hypocotyls elongated, the hypocotyls and leaves were aseptically detached and cultured on solid, full-strength MS media supplied with the same constituents as described above and placed at 23±℃ in the

dark for approximately 3 to 6 weeks. Thereafter, the callus was periodically transferred every four weeks to a new medium with the same composition to increase proliferation and to maintain its growth.

3.2.3 Statistical Analysis

Unless otherwise stated, experimental data were analysed using the STATISTICA Version 8 (StatSoft Inc. 2005) package. Where applicable, one way analysis of variance (ANOVA) was carried out to infer differences between treatments. All percentage data was arcsine transformed to normalize the data before carrying out the ANOVA at the 95% confidence level. When the ANOVA proved existence of a significant difference between samples, Fisher's LSD Test was carried out as *a post-hoc* test. The return of a *P*-value < 0.05 was regarded as significant.

3.3 Results and Discussion

The leaf and hypocotyl explants of the wild-type Columbia-O, *max1-1*, *max2-1*, *max2-1*, *max3-9* and *max4-1* mutants of *Arabidopsis thaliana* were cultured on MS media with two combinations of auxin and cytokinin concentrations (2:2 [mg/L] and 0.5:0.05 [mg/L] 2,4-D:kinetin). The first hormone concentration (2:2 mg/L 2,4-D: kinetin) was selected based on a previous pilot study that took place within the IPB, whereas the latter concentration was chosen based on literature (Huang and Yeoman, 1984). These different concentrations of 2,4-D and kinetin were tested in order to identify the best concentration for good quality callus. The analysis of data took place after 5 to 6 weeks of culture. Generally, the *Arabidopsis* seeds of all the genotypes placed on the callus induction medium started to germinate and etiolate within 3-5days. This result is in agreement with the observations made by Huang and Yeoman (1984). Within 14 days, the calli started to appear from the edges and then spread throughout the explant. This is in agreement with Huang and Yeoman (1984) and Amiri *et al.* (2011) who observed the same pattern of callus induction in *Datura stramonium.*

The effects of the genotypes on callus induction from *Arabidopsis thaliana* seeds are shown in Table 3.1. Callus induction in *Arabidopsis* varied according to the genotype used. Among the six studied genotypes, *max1* was the most efficient in producing calli for both hormone combinations. This was also true for the callus produced by the cotyledons (Data not shown). The genotypic mean percentage callus induction ranged from 34.44 to 93.33%. The *max1* mutant initiated callus earlier than other genotypes, results were observed in about two to six weeks after culture. Furthermore, *max1* resulted in a cream-white (Table 3.2), compact and very bulky callus. The *max2* and *max4* genotypes were no different in appearance from the wild type, which was cream, soft, and compact under both hormone combinations. These mutants, however, produced a lesser amount of callus compared with the wild-type and *max 1.*

Table 3.1 Effect of genotype and hormone concentration on callus induction from hypoctyl explants. (The values are the means ± SE).

Genotype	Mean Frequency of Callus Induction (%) ± SE	
	0.5:0.05 mg/L 2,4-D: kinetin	2:2 mg/L 2,4-D:kinetin
max1-1	92.22±3.18 [c]	83.33±5.09 [b]
max2-1	58.88±6.31 [b]	50.00±7.65 [ab]
max2-2	46.677±7.10 [ab]	36.67±5.09 [a]
max3-9	34.44±5.56 [a]	40.00±6.89 [a]
max4-1	42.22±5.88 [a]	47.78±5.28 [a]

Different letters in the same column indicate values that were determined by ANOVA (Kruskal-Wallis) to be significantly different ($P < 0.05$) from each other. Data for the wild-type was not recorded.

Table 3.2 The response of different *Arabidopsis thaliana* genotypes on two combinations of 2,4-D and kinetin in callus formation from hypocotyl explants

Treatment 2,4-D:Kinetin (mg/L)	Genotype	Callus initiation-days	Callus characteristic
0.5:0.05	*max1-1*	14 – 15 a	Compact, cream to white
	max2-1	14 – 15 a	Compact, cream
	max2-2	14 – 15 a	Friable, cream
	max3-9	>21 b	Compact, cream to yellow
	max4-1	> 21b	Compact, cream
2:2	*max-1*	14 – 15 a	Compact, white
	max2-1	14 – 15 a	Compact, cream
	max2-2	14 – 15 a	Compact, cream
	max3-9	>21 b	Compact, cream to yellow
	max4-1	>21 b	Compact, cream

Different letters indicate values that were determined by ANOVA (Fisher LSD test) to be significantly different ($P < 0.05$) from each other. Data for the wild-type was not recorded.

The colour exhibited by *max3* callus was dark cream to slight brown at maturity (Figure 3.1). Callus for this mutant was also compact and less bulky compared with the other genotypes. This genotype, like *max4*, also took slightly longer to form calli compared with the other genotypes. The genes disrupted in the two mutants encode carotenoid cleavage dioxygenase enzymes, which play a role in strigolactone biosynthesis (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). When the response of each genotype was analyzed per hormone combination used, a similar response was obtained. Thus no apparent differences were observed, meaning that both hormone combinations used are suitable for *Arabidopsis thaliana* callus formation with more or less the same potential of triggering callus induction.

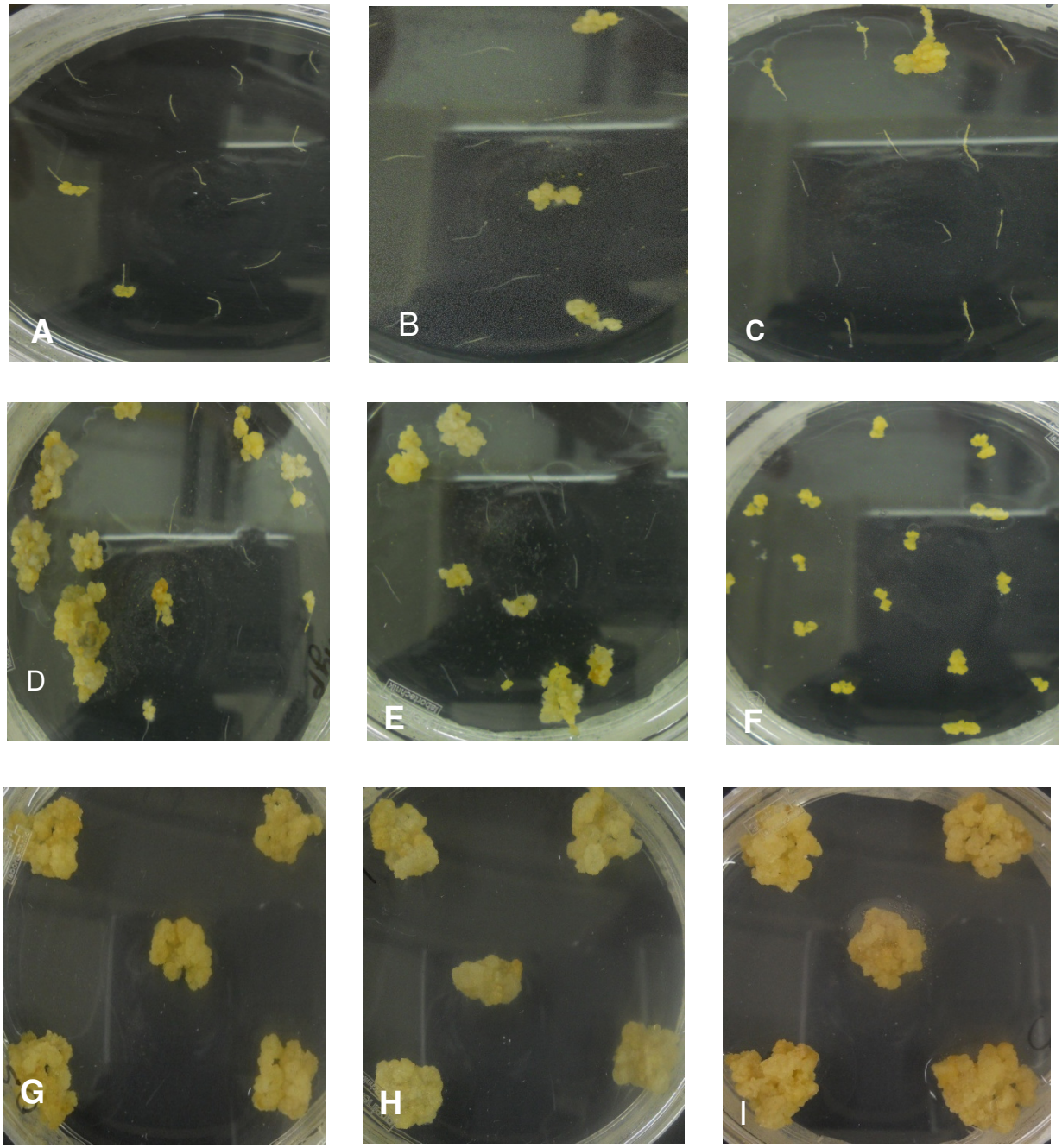

Figure 3.1 Callus initiation from hypocotyl explants. Calli are shown at three weeks (A-C) and six weeks (D-F) post induction, as well as at a mature stage after several rounds of sub-culturing (G-I). Calli start to appear from the edges and spread throughout the entire explant. (A) *max1* [0.5:0,05 mg/L 2,4-D:Kinetin],(B) *max2-2* [0.5:0.05 mg/L 2,4-D:Kinetin] (C) *max2-2* [2:2 mg/L 2,4-D:Kinetin], (D) *max2-1* [0.5:0.05 2,4-D:kinetin], (E) *max2-2* [2:2 mg/L 2,4-D:Kinetin], (F) *max3-9* [2:2 mg/L 2,4-D:Kinetin], (G) Columbia-O [0.5:0.05 2,4-D:kinetin], (I) mature *max 3-9* callus showing slight colour change from yellow to slightly brownish.

Rapid proliferation of the callus occurred, with the callus being sub-cultured to a new medium of the same composition every four weeks. Light has been reported to significantly increase the mass of calli of various species (Chawla, 2002). However, calli grown in the presence of light have a tendency of producing poly-phenolic compounds which cause browning that might consequently lead to death of the callus (Chawla, 2002). This is usually an oxidation process that is highly favoured by light and may explain why there is less browning in the dark (Chawla, 2002). Due to these facts, calli in this study were grown in constant darkness. The best growing single callus from each genotype was selected after 6-8 weeks and placed on fresh medium. This callus was allowed to grow and was sub-cultured periodically, which accelerated growth giving rise to an abundant callus stock in 4-5 months which was utilized in experiments described in Chapters 4 and 5. Although *max3* and *max4* initiated callus, their percentage induction was very low (34.44% and 42.22% [0.5:0.05 mg/L 2,4-D:Kinetin]; 40% and 47.78% [2:2 mg/L 2,4-D:Kinetin]) respectively, compared with other genotypes (Fig 3.1). This observation could be due to differences in endogenous hormone (auxin and kinetin) levels amongst the genotypes. A strong hypothesis associates genotypic differences during *in vitro* culture with differences in endogenous hormone levels (Mok and Mok, 1977; Henry *et al*., 1994; Pellegrineschi, 1997). Although *max3* and *max4* showed lower callus initiation efficiencies, their growth (size and type) was not affected. All observations described above were also observed for the cotyledon explants (data not presented).

3.4 Conclusion

Suitable callus was attained from media containing both combinations of hormones from both the hypocotyl and leaf explants. The callus formed was used to investigate its response to smoke and GR24 treatments, which are discussed in the next two chapters. Thus an efficient method to produce callus for further investigations of our research was established in the present study.

3.5 References

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Chapter 4

The effects of strigolactones and smoke in *Arabidopsis thaliana* callus cultures

4.1 Introduction

Plant growth and development include the integration of various environmental and endogenous cues that, together with intrinsic genetic programs, define plant form (Gray, 2004; Wolters and Jurgens, 2009). The plant hormonal system is presumed to play a major role in the control of growth and development (Lincoln *et al.,* 1990). There is strong evidence that suggests that the environmental cues target perception or biosynthesis of phytohormones (Depuydt and Hardtke, 2011). This orchestrates intrinsic developmental programs and simultaneously transduces environmental inputs (Depuydt and Hardtke, 2011). Phytohormones are central for growth regulation in a number of plants. Various plant processes can be modulated by phytohormones in a sometimes synergistic manner, indicating some cross talk between different pathways. It is still not clear whether phytohormones target common or different transcriptome modules (Depuydt and Hardtke, 2011).

Auxins and cytokinins have been shown to mediate various plant growth and developmental processes, especially bud outgrowth, in an antagonistic manner (Muller and Leyser, 2011). Significant progress in plant growth and development studies has resulted in the recognition of smoke and strigolactones as important regulators of plant growth and development (Nelson *et al*., 2011), as described in Chapter 2.

The effects of smoke on seed germination were first discovered by De Lange and Boucher (1990). The application of smoke, or its extracts, in various processes of plant growth, including seed germination and seedling vigour, of a number of plants has received considerable attention (Modi, 2002; 2004; Baxter and Van Staden,

1994; Blank and Young, 1998; Sparg *et al.*, 2005; Sparg *et al.*, 2006; Jain and Van Staden, 2006; Kulkarni *et al.*, 2006; Van Staden *et al.*, 2006; Kulkarni *et al.*, 2007).
A landmark discovery recently identified strigolactones as the major regulators of shoot branching (Umehara *et al.*, 2008, Gomez-Roldan *et al.*, 2008). Shoot branching has been defined as one of the major determinants of the overall plant form and function (Finlayson, 2007). It contributes greatly to the diversity of growth habits occurring within and between species. This branching feature of plants is also relevant to modern agriculture, due to the fact that maintaining a considerable branching habit increases the use of resources as well as the production of biomass and reproductive organs (Finlayson, 2007). The structural similarity between smoke and strigolactones has garnered considerable attention. It has been shown that KAR_1, the major biologically active compound in smoke, was able to substitute for strigolactones in the germination of the parasitic plants *Striga* and *Orobanche* (Nun and Meyer, 2005; Daws *et al.*, 2007). However, this report has since been debated due to the results of Nelson *et al.* (2010), where it was shown that KAR_1 was unable to trigger germination of *Orobanche minor,* whereas GR24 was active at very low concentrations, suggesting different modes of action or species-specificity for response to these two compounds.

The growth promoting effects of smoke-water or its extracts in plants, including callus bioassays (Jain *et al.*, 2008) have been widely documented. Investigations into biomass accumulation following GR24 treatment have recently emerged (Kotze, 2010; Steenkamp, 2011). Callus cultures are an alternative source to whole plants systems for studying responses of plant cells to various physiological and biochemical factors (Sharathchandra *et al.*, 2011). Callus cultures are independent of geographic, seasonal variations and changing environmental factors. The influence of remote plant parts is excluded when using callus cultures. The aseptic environment minimizes the interfering of pathogens and insects that could be detrimental to the growth of plants (Smeda and Weller, 1991). Plant cultures can also be manipulated to undergo biotransformation for manufacture of desirable compounds (Vijaya *et al.*, 2010). Additionally, there are high probabilities that the whole plant penetration and transduction barriers which are capable of interfering with the movement and absorption of exogenously applied plant growth regulators to

the site of action are absent in cell cultures (Smeda and Weller, 1991). The short regeneration time of cultured cells allows rapid data collection and reproducibility (Smeda and Weller, 1991).

The main objective of this particular chapter was to use *Arabidopsis thaliana* callus, for the reasons given above and for the genetic resources available (as explained in Chapter 2), to help in the determination of the molecular basis for the growth promoting effects of smoke and GR24. Additionally, no biomass accumulation studies following GR24 treatments have been documented on callus bioassays. Due to the structural similarities and the broad range of plant growth and development mediated by karrikins and strigolactones, it would be interesting to determine whether smoke-water and GR24 would trigger similar effects on the growth of *Arabidopsis thaliana* callus cultures.

4.2 Materials and Methods

4.2.1 Plant material

Callus that was previously grown at hormone concentrations of 2:2 mg/L (C1) and 0.5:0.05 mg/L (C2) 2,4-D and kinetin, as previously described in Chapter 3 (Section 3.2.1), was used in all experimentation described in this chapter. All lines used were in the Col-O background and included *max1-1*, *max2-1*, *max2-2*, *max3-9* and *max4-4*, as described in the previous chapter.

4.2.2 Plant growth promoting substances

The aqueous smoke solution used in all experiments was donated by Prof J van Staden from the Research Centre for Plant Growth and Development, University of KwaZulu-Natal, Pietermaritzburg. The aqueous smoke solution was prepared according to a method described in Baxter *et al.* (1994) and was further stored in a foil-covered glass bottle at 4°C. The synthetic strigolactone analogue, GR24 was obtained from Prof B Zwanenburg of the Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands.

The specific GR24 concentration used in the experiments ($1x10^{-7}$ M) was selected as a result of previous experiments conducted at the Institute for Plant Biotechnology (IPB), Stellenbosch University (Kotze, 2010). All media containing smoke was autoclaved separately to the control and GR24-containing media to avoid cross-contamination from any volatile smoke compounds. This ensured that the growth promoting activities of the smoke were excluded from all other media.

4.2.3 Growth conditions

In order to determine the appropriate callus mass to be used in this study, two different callus masses of 5 mg (W1) and 50 mg (W2) respectively, were selected and screened. Calli were fully weighed, under aseptic conditions, and their masses recorded. Five calli were plated per 90 x 10 mm diameter growth plate (Greiner Bio-One, Cellstar®) and each replicate consisted of 5 plates (n=25). The media used were identical to those used for subculture of the callus (Chapter 3), except for the addition of the respective plant growth promoting substances. Smoke water was applied at a dilution of 1:1000 (based on a smoke dilution series experiment as described in section 4.3.2), whilst GR24 was added to a concentration of $1x10^{-7}$ M, as was previously optimized within the IPB (Kotze, 2010). For the auxin or cytokinin independent assay, media was prepared and supplemented with smoke-water or strigolactone in the presence of either 2,4-D or kinetin alone. In another assay (AC-free), callus was grown on MS media that lacked both auxin and cytokinin but supplemented with either smoke-water or strigolactones. Thus the media used were identical to those used for sub-culturing (see Chapter 3), except for the exclusion of the auxin (2,4-D) and cytokinin (kinetin). The growth promoting substances were added to the media before adjusting the pH to 5.8 with KOH. Gelrite (2.2 g/L) was then added to the media prior to autoclaving at 121°C, 100 kPa for 20 minutes. The growth plates with calli were incubated in the dark at 23±2°C for 28 days. Following the growth period, the calli were carefully lifted off the media and gently blotted dry before weighing each callus mass. The calli were then dried in an oven at 70 °C for three days to determine the dry mass. All experiments were independently repeated at least three times. Relative growth rate was calculated from the fresh mass,

whereby mean mass increase was determined and expressed as percentage of the largest increase.

4.2.4 Statistical Analysis

Experimental data were analysed using the STATISTICA Version 8 (StatSoft Inc. 2005) package. Where applicable, factorial analysis of variance (ANOVA) to infer differences between treatments was carried out. All percentage data was arcsine transformed to normalize the data before carrying out the ANOVA at the 95% confidence level. When the ANOVA proved existence of a significant difference between samples, Fisher's LSD Test was carried out as a *post-hoc* test. The return of a *P*-value < 0.05 was regarded as significant.

4.3 Results and Discussion

4.3.1 Determination of the appropriate callus mass for experimentation

In order to determine the appropriate amount of callus to use for further experimentation, 5 mg and 50 mg calli were placed onto C1 and C2 media containing $1x10^{-7}$ M GR24. Both the 5 mg and 50 mg masses of callus lead to significant mass increases in both hormone concentrations used, indicating that growth had taken place (Figure 4.1). Interestingly, both masses resulted in a clear indication of the differences between the GR24-treated and the control calli. The higher initial mass (W2) generally resulted in greater growth ($p<0.005$) for both the control and GR24 treatments, regardless of the combination of auxin and cytokinin used. This suggests that the greater the initial mass of the callus, the more biomass will accumulate. Furthermore, slight differences between W1 and W2 callus could be observed visually. For instance, the W2 callus had a healthier and whiter appearance compared with that of W1. Accordingly, W2 was selected to further establish the growth effects of GR24 and smoke in the tissue culture system.

Initial variability studies by Briggs and Kidd (1920) on the seeds of *Helianthus annus* species used five different classes of seeds, based on their initial air-dry mass. In an open field, an equal number of seeds from each size class were sown. Plant

samples grown from seeds of each size class were then harvested periodically with the determination of the leaf areas from each class of all the samples. The leaf area and the mean dry weight were expressed as a percentage of the largest. The leaf areas from each class of all samples were determined. The results revealed a correlation between the initial seed mass and the mean plant mass, suggesting that the bigger the seed, the larger the plant will arise from it (Evans, 1972). This could possibly explain why W2 in this study resulted in more growth (the greater the initial mass of the callus the greater is biomass accumulation).

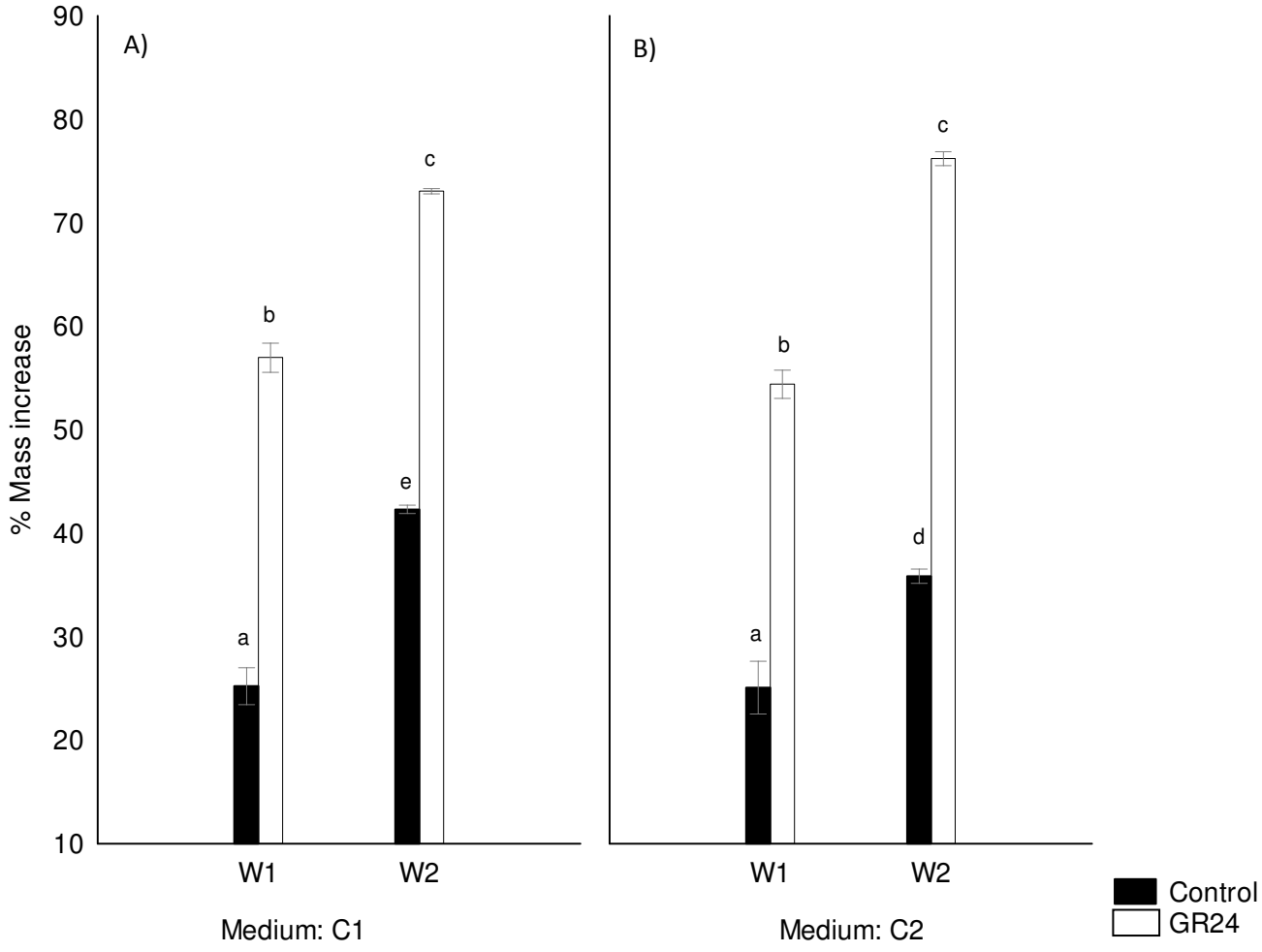

Figure 4.1 Growth results of the masses of callus W1 (5mg) and W2 (50 mg) grown on MS medium containing the two different hormone combinations: 2:2 mg/L 2,4-D:kinetin (C1) (A) and 0.5:0.05 mg/L 2,4-D:kinetin (C2) (B). Both media were supplemented with $1x10^{-7}$ M GR24. Different letters indicate values that were determined by ANOVA (Fisher LSD test) to be significantly different ($P < 0.05$) from each other.

In many phenomena of nature there are some processes whereby the rate of change of some quantity is proportional to the quantity itself. For instance, in the case of ordinary plant, the leaf area will increase as growth proceeds (Blackman, 1919). Consequently, the increasing leaf area will cause the rate of production of material by assimilation to increase resulting in more rapid growth. This in turn gives rise to a greater leaf area and more production of assimilating material (Blackman, 1919). Due to the fact that money paid out at compound interest follows this trend, whereby the increase rate is at any time proportional to the amount of capital, this phenomenon was named the compound interest law by Lord Kelvin. It was only in 1917, that this law was applied in plant growth after Gregory's plant growth experiments on cucumber (Blackman, 1919). Collectively these observations explain why the mass of W2 was significantly more than the mass of W1.

4.3.2 Screening for the appropriate smoke concentration

In an attempt to identify the appropriate concentration at which the smoke-water would be effective in this callus system, different dilutions were tested. These ranged from 1:500 to 1:5000. The mass of the callus was significantly increased at the 1:500 and 1:1000 dilutions on media containing 2:2 mg/L 2,4-D: kinetin (Fig 4.2A). This result was consistent with previous research conducted at the IPB. For media containing 0.5:0.05 mg/L 2,4-D:kinetin, all smoke dilutions resulted in a higher mass increase than the control, except for 1:5000 (Fig 4.2B) Clearly, 1:1000 was the best dilution for both hormone combinations investigated, therefore based on these results, 1:1000 was selected as the best dilution for both hormone combinations and it was further employed, when necessary, in all further experiments.

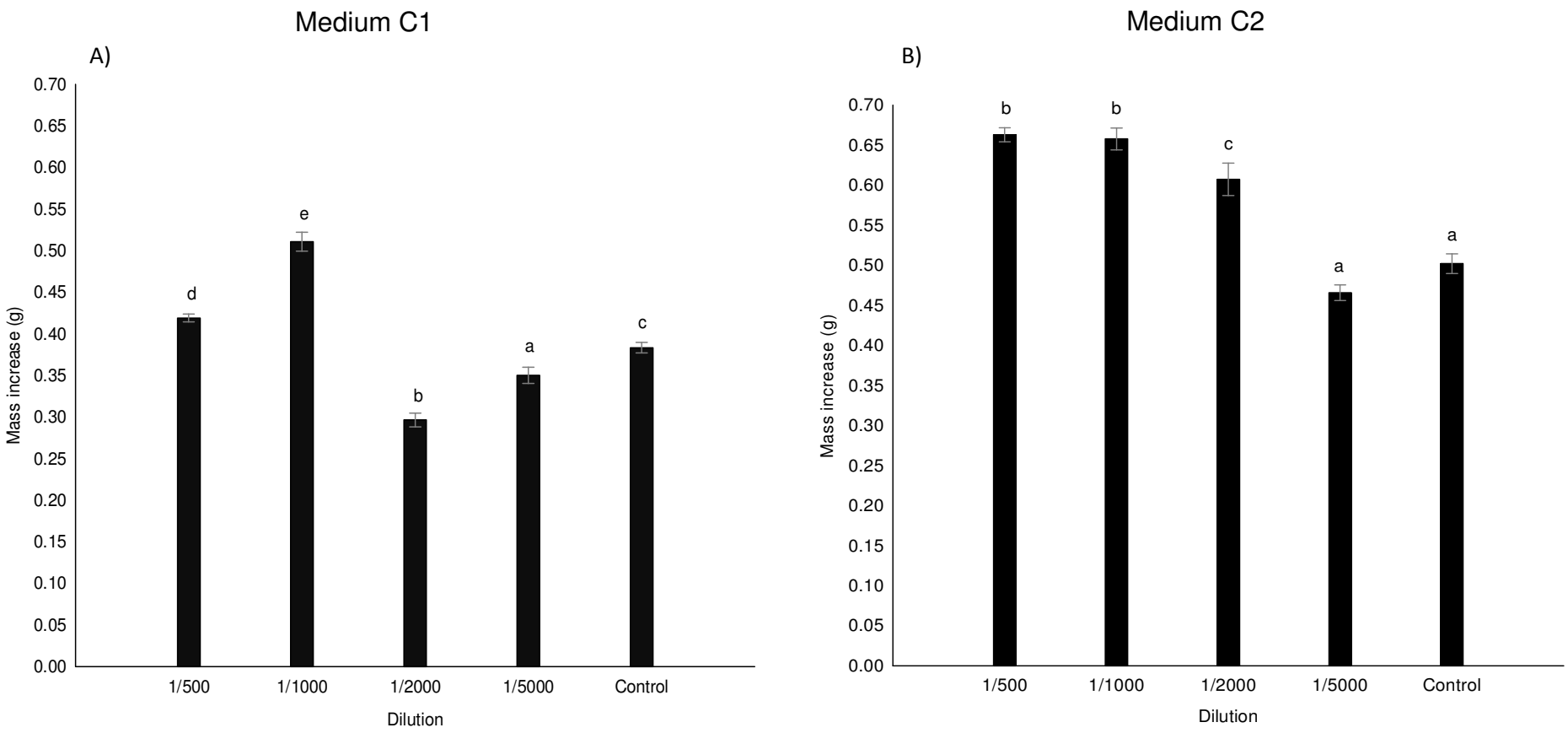

Figure 4.2 Mass increase in *Arabidopsis thaliana* callus treated with a dilution range of smoke-water on MS medium containing 2:2 mg/L 2,4-D:kinetin (A) and 0.5:0,05 mg/L 2,4-D:kinetin (B). Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different ($P < 0.05$) from the control.

4.3.3 Smoke-water and GR24 promote the growth of callus in the presence of both auxin and kinetin

Significant differences between the controls and the strigolactone and smoke-water treatments were obtained for media with both hormone combinations C1 and C2 ($p<0.05$) (Fig 4.3A). Generally, the smoke-water treatment resulted in a higher mass increase compared with GR24 at both hormone combinations. The mean growth increase was higher at the C2 hormone combination as compared with the C1 combination. Therefore, in this system, callus that had been previously grown at low hormone concentrations and then treated with GR24 and smoke-water produced more biomass than callus grown at high hormone concentrations, suggesting that the ratio of hormones applied affects the response of callus to GR24 and smoke-water treatments. The dry mass results (Fig 4.3B) correlated with the above results, whereby smoke and GR24 resulted in increased growth compared with the control at both hormone combinations. The low hormone combination (C2) still resulted in a greater mass increase than the higher combination (C1), with smoke being the best treatment in terms of biomass accumulation for both combinations of hormones.

In this study, application of smoke-water promoted the growth of *Arabidopsis thaliana* callus compared with the controls (Figure 4.3). In a similar study, application of smoke-water on tomato plants promoted the accumulation of biomass in addition to enhanced germination and seedling vigour compared with controls (Kulkarni *et al*., 2008). The smoke-treated plants also fruited earlier and yielded higher numbers of fruit than controls, a very important aspect in horticulture (Kulkarni *et al*., 2008; Kulkarni *et al*., 2011). Additionally, foliar application of smoke-water and butenolide on tomato young tomato seedlings demonstrated a significant improvement in growth (Kulkarni *et al*., 2007). In another study, maize plants that were soaked in smoke-solution showed more leaves than their respective controls (Van Staden *et al*., 2006).

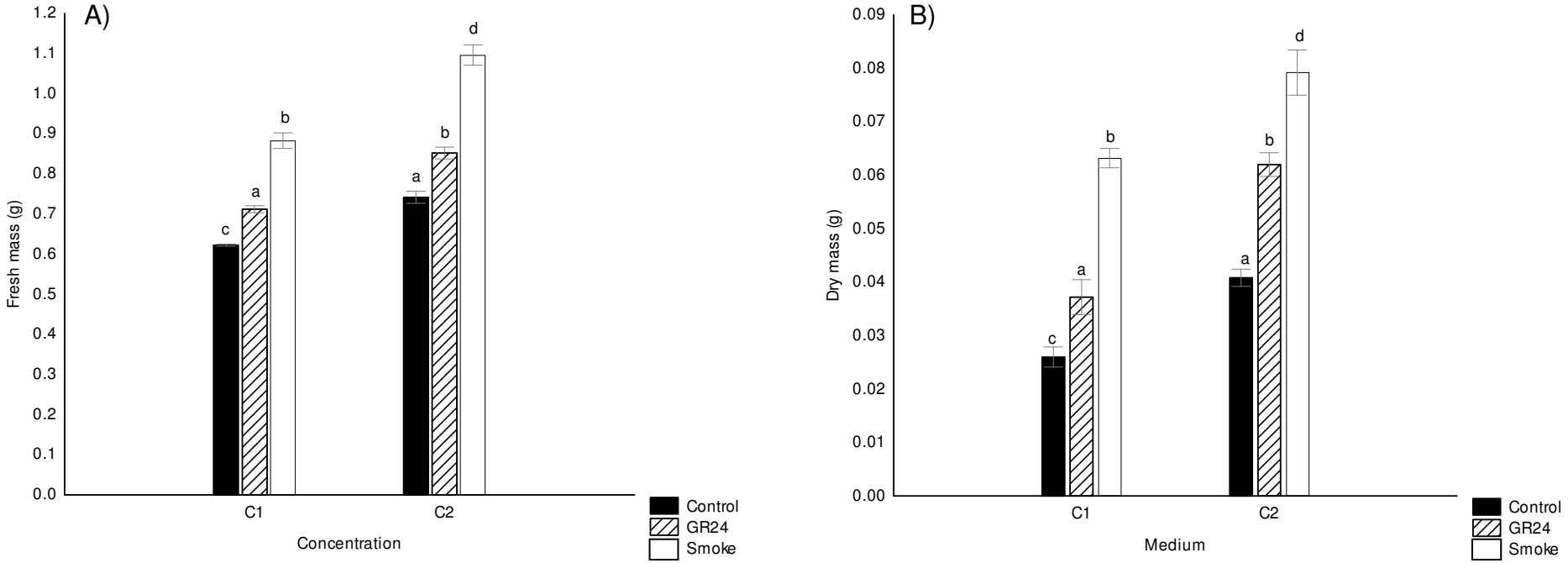

Figure 4.3 The effects of smoke-water and GR24 on the growth of *Arabidopsis thaliana* callus. (A) Fresh mass and (B) dry mass of *Arabidopsis thaliana* callus on MS medium containing 2:2 mg/L 2,4-D:kinetin (C1) and 0.5:0,05 mg/L 2,4-D:kinetin (C2), supplemented with 1:1000 smoke or $1x10^{-7}$ M GR24 .

Furthermore, seeds of Australian bush tomato demonstrated enhanced germination, which was related to increased productivity, after treatment with smoke-water (Ahmed *et al*., 2006). Recently, smoke-water and KAR_1 treatment showed stimulatory effects on the growth of onion plants. A number of growth parameters such as leaf length, leaf fresh and dry weights and number of leaves were significantly promoted in treated plants over their controls. During harvest, smoke-water and KAR_1 treated plants resulted in larger onion bulbs without any toxic effects (Kulkarni *et al*., 2010). These results indicate that smoke-water/extracts have a potential to improve biomass accumulation in a number of plants.

The ability of smoke to regulate the growth of cell cultures has also been demonstrated. For instance smoke treatment enhanced the embryogenic potential of a hypocotyl culture of geranium plant. The rate of embryo development on the smoke-treated culture was at a faster rate compared to the controls (Senaratna *et al*., 1999). Another growth regulatory role of smoke in cell cultures was shown using soybean and mungbean cultures. Smoke-derived karrikin affected the mass of soybean callus in a concentration dependent manner, whilst it stimulated root initiation and development in mungbean hypocotyls (Jain *et al*., 2008). These findings clearly indicate that cell cultures react positively to smoke-water treatments.

Strigolactone (GR24) application significantly improved the growth of *Arabidopsis thaliana* callus in this study. Interestingly, when studying the response of *Nicotiana benthamiana* seedlings to GR24 and KAR_1, Kotze (2010) observed an increase in seedling vigour. Steenkamp (2011) showed that smoke and GR24 treatments enhanced the mass, length and leaf area of *Nicotiana benthamiana* seedlings when grown under normal conditions. However, under salinity conditions, smoke treatment significantly increased lateral root number in addition to increased fresh mass, root length and number of leaves. When GR24 treated seedlings were grown under the same conditions, fresh mass, leaf number and lateral root number were significantly increased. Marginal increases in root length and leaf area were also observed. These findings suggested that strigolactones may positively enhance crop yield.

This is the first study to report on the promotion of biomass accumulation of *Arabidopsis thaliana* callus cultures through exogenous application of strigolactones.

In addition to recent reports on repression of shoot branching by strigolactones, a novel role for strigolactones in plant growth has been demonstrated.
From the data (Figure 4.3) it is apparent that both smoke-water and GR24 increased the biomass yield of the callus cultures. The growth promoting activities shown by smoke and GR24 in this study could possibly be due to their structural similarities as described in Chapter 2. Strigolactones and karrikins signalling might be mediated via the F-box protein, MAX2. Hence, it is possible that the growth promoting effects of smoke and GR24 in this study occurs through the MAX2 signaling pathway. This was tested and is discussed below (Section 4.3.7).

4.3.4 Strigolactones and smoke-water promote growth in the absence of cytokinin

On media containing only 2,4-D and no cytokinin, callus that was previously grown on MS media supplemented with 2:2 mg/L 2,4-D:kinetin (C1), resulted in increased growth when treated with GR24 or smoke-water compared with the control (Fig 4.4.A). However, the smoke-water treatment was not significantly different from the control ($p>0.05$) although its mean mass increase was higher than that of the control. Contrarily, smoke-water treatment resulted in increased growth compared with GR24 and the control (not significantly different from one another) when applied to callus that was previously grown on C2 medium (0.5:0.05 mg/L 2,4-D:kinetin).

When grown on medium containing only kinetin and no auxin, callus that was previously grown on C1 medium resulted in no significant differences in mass ($P>0.05$) between the smoke-water, GR24 and control treatments (Figure 4.5). Growth of the callus that was previously grown on C2 medium was strongly decreased with GR24 and smoke treatments (Figure 4.5). Generally, it was noted that the mass of the callus grown on the kinetin-only system (kinetin only, kinetin + GR24 or Kinetin + Smoke-water) was very low compared with that of the callus grown on the 2,4-D system. Differences on the callus grown on these two systems were also observed by visual analysis. For instance, the callus grown in the presence of 2,4-D had a very healthy appearance with large clumps (Figure 4.6). On the other hand, callus that was grown on media supplied with kinetin had a dark-yellow, unhealthy appearance, regardless of GR24 or smoke supplementation (Figure 4.7).

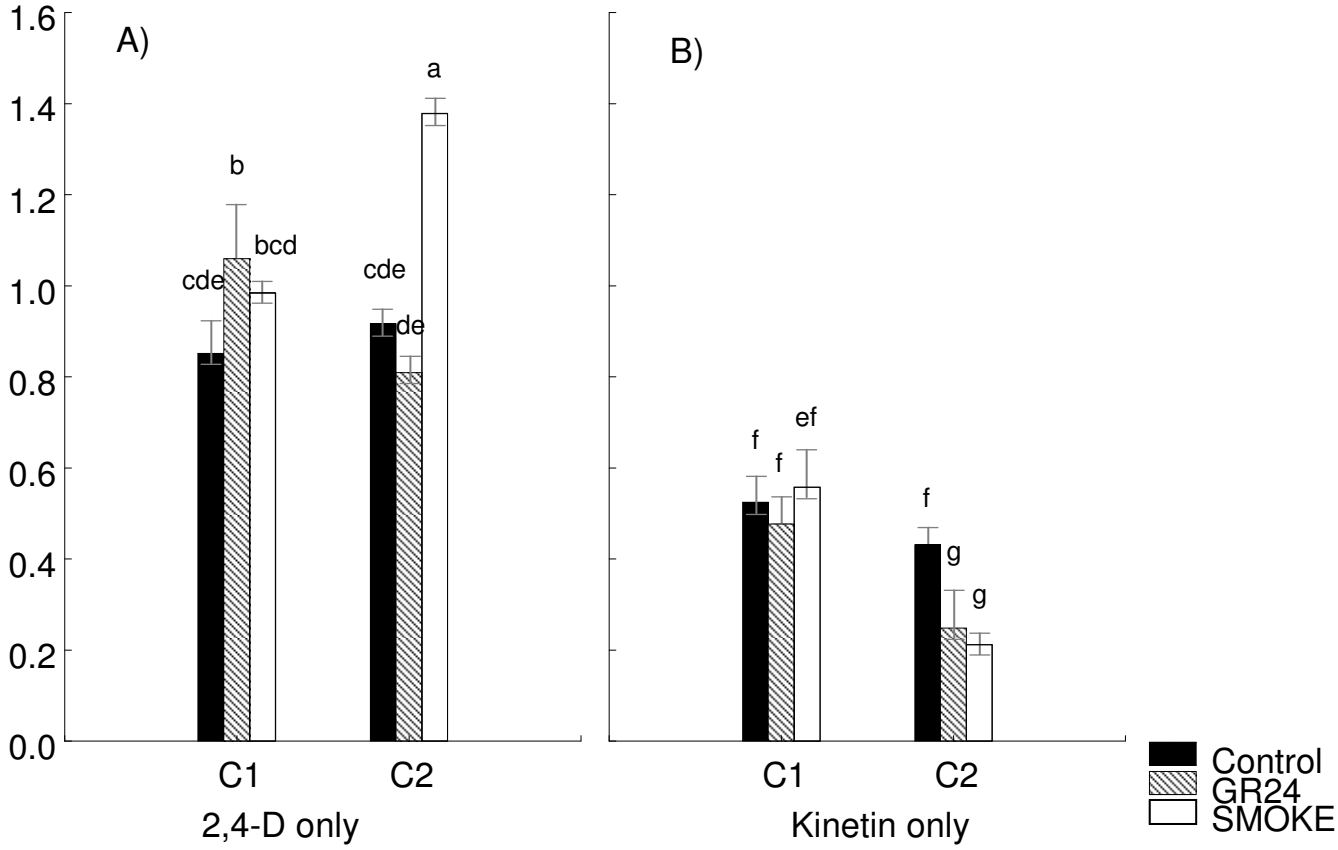

Figure 4.5 Mass increase of *Arabidopsis thaliana* callus treated with GR24 or smoke-water on 2,4-D only (A) or kinetin-only (B) medium. C1 and C2 indicate the medium on which the callus had been sub-cultured. The concentrations of 2,4-D and kinetin only medium were same as for the respective subculture media. Different letters indicate values that were determined by ANOVA (Fisher LSD test) to be significantly different ($P < 0.05$) from each other.

The positive growth stimulatory effect shown by the combination of 2,4-D and GR24 or smoke is not surprising as several findings suggested a synergistic relationship between auxins and strigolactones (Brewer *et al*., 2009; Dun *et al*., 2009; Ferguson and Beveridge, 2009; Hayward *et al*., 2009). This interaction plays a major role in shoot branching control, as described in Chapter 2.

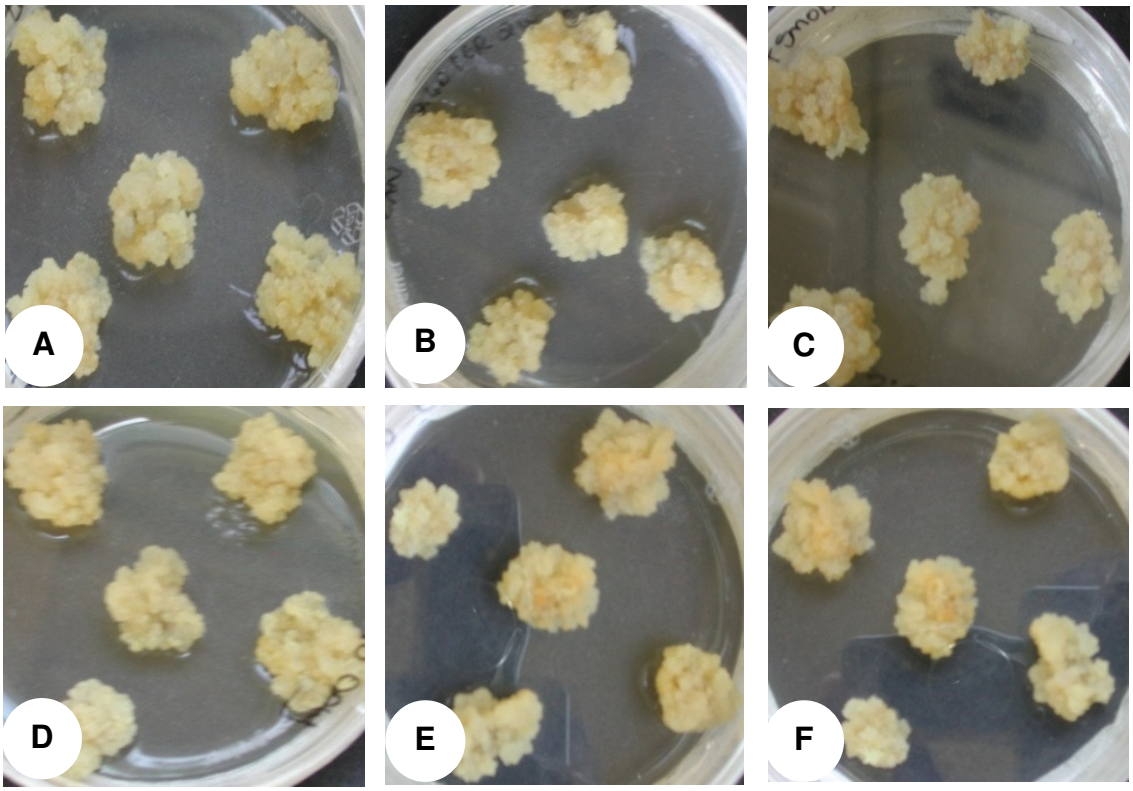

Figure 4.6 Callus growing on MS medium supplemented with 2,4-D only (A and D), 2,4-D + GR24 (B and E) and 2,4-D + smoke-water (C and F). (A)-(C) show callus that was previously grown on MS medium supplemented with 2:2 mg/L 2,4-D:kinetin (C1), (D)-(F) show callus that was previously grown on MS medium supplemented with 05:0.05 mg/L 2,4-D:kinetin (C2). The calli appear healthy and formed large clumps.

The importance of auxins for initiation and growth of callus of a number of plant species is well documented. Studies have indicated that, the synthetic auxin 2,4-D is more essential for callus growth than cytokinin and is capable of promoting growth on its own (Sadhu, 1974; Sharma *et al.,* 1981). In this study, the presence of auxin, without cytokinin, was sufficient to allow for enhanced biomass accumulation in callus following treatment with GR24 and smoke water, although the concentration of the auxin influenced the effects of these substances on the callus growth (Figure 4.4 and 4.5A).

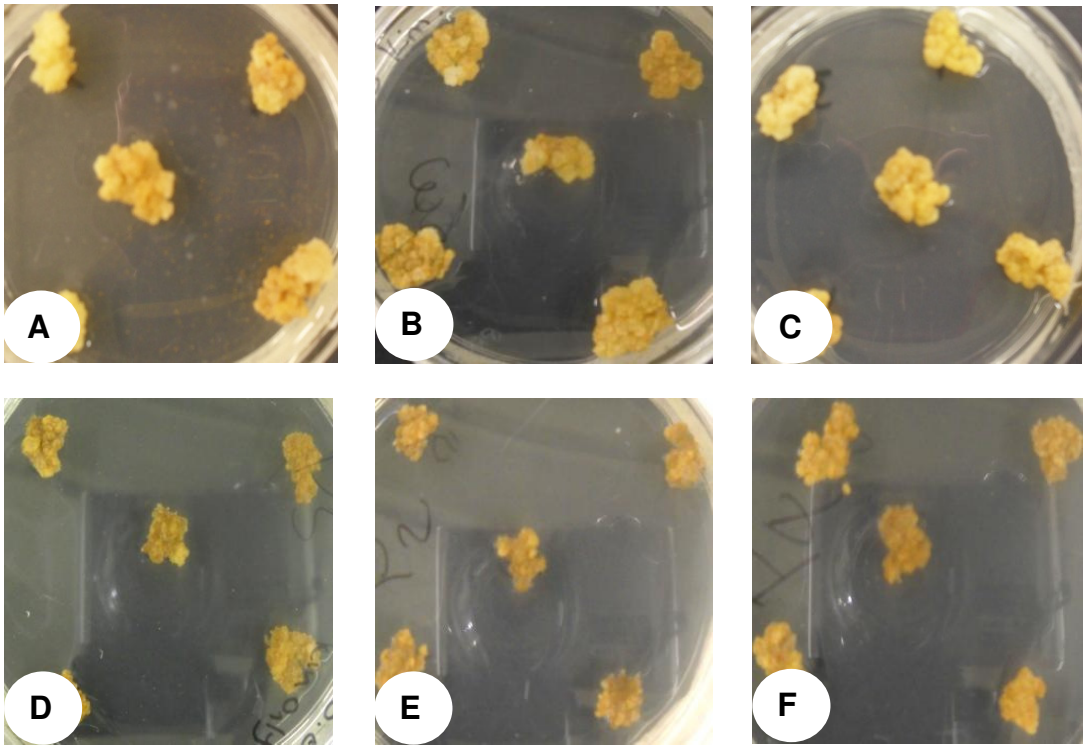

Figure 4.7 Callus growing on MS medium supplemented with kinetin only (A and D), kinetin + GR24 (B and E) and kinetin + smoke-water (C and F). (A)-(C) show callus that was previously grown on MS medium supplemented with 2:2 mg/L 2,4-D:kinetin (C1), (D)-(F) show callus that was previously grown on MS medium supplemented with 0.5:0.05 mg/L 2,4-D:kinetin (C2). All calli exhibit a very dark yellow phenotype.

Kinetin is one of the most extensively used hormones in tissue culture (He and Lazzeri, 2001). Several authors have reported on the inefficiency of cytokinin to induce callus formation without auxin. In this study, kinetin alone was not sufficient for callus growth and it supressed the growth promoting effects of strigolactone and smoke (Figure 4.4 B). This result is not surprising, given the fact that strigolactones and cytokinins act antagonistically on shoot branching control. Both classes of compounds are mainly produced in the root but also locally in the shoot and are transported acropetally in the xylem. However, cytokinin moves acropetally to promote bud outgrowth, whereas strigolactones move acropetally to inhibit bud outgrowth (Cline, 1991; Napoli, 1996; Foo *et al.,* 2001; Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Therefore, the reduction in growth observed on when growing the callus in the presence of cytokinin and strigolactones without auxin could be attributed to these antagonistic effects.

4.3.5 Strigolactone treatment promote biomass accumulation independently of exogenously-applied auxin and cytokinin

Treatment with GR24 promoted an increase in fresh biomass of calli from both sub-culture conditions on AC-Free media, whilst smoke water did not result in any differences in growth compared with the controls ($P < 0.05$) (Fig 4.7A). Similar results were observed for the dry mass measurements, although smoke water treatment resulted in a significant decrease in fresh mass in calli previously grown on 0.5:0.05 mg/L 2,4-D:kinetin (Fig 4.7B). Generally, callus that was previously grown at a hormone concentration of 2:2 mg/L 2,4-D:kinetin had significantly higher mass ($P < 0.05$) compared with callus that was previously grown at the lower hormone concentration (0.5:0.05 mg/L 2,4-D:kinetin). Additionally, dry mass measurements showed a similar growth trend (Fig 4.7B).

From the data (Figure 4.7), it is clear that strigolactone was able to promote the growth on its own (no exogenously-applied auxin or cytokinin). On the other hand, smoke cannot promote growth without the addition of auxin and cytokinin. The growth stimulatory effect of smoke-water on *Arabidopsis thaliana* callus seems to be strongly reliant on the presence of auxin in the medium. This observation is also not surprising as it has been reported that there are many factors that contribute to the growth of callus, including the concentration of plant growth regulators (Tang *et al*., 1997).

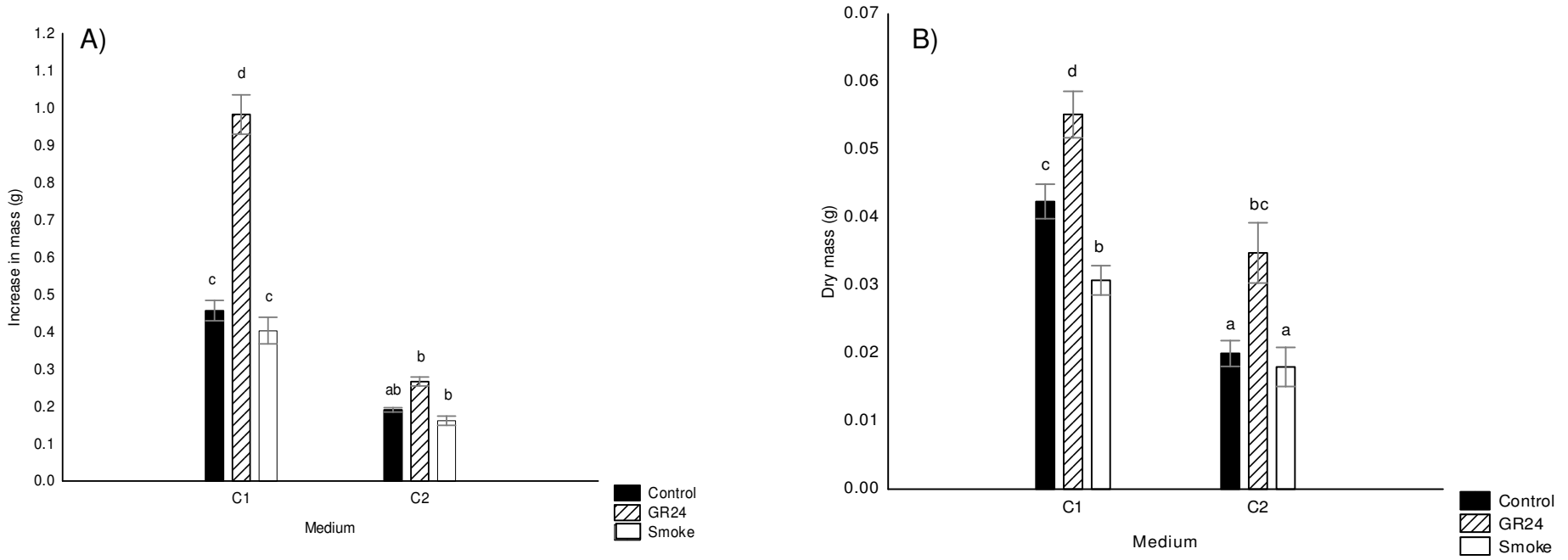

Figure 4.8 Fresh mass increase (A) and dry mass (B) of *Arabidopsis thaliana* callus which was previously grown on MS medium containing either 2:2 mg/L 2,4-D:Kinetin (C1) or 0.5:0.05 mg/L 2,4-D:Kinetin (C2). The callus was transferred to AC-Free MS medium supplemented with $1x10^{-7}$ M GR24 or 1:1000 smoke-water. Different letters indicate values that were determined by ANOVA (Fischer LSD test) to be significantly different ($P < 0.05$) from each other.

4.3.7 Biomass accumulation is mediated via MAX2

In order to investigate whether strigolactone signalling is important for the biomass accumulation in response to GR24 and smoke-water observed in this study, calli of the *Arabidopsis more-axillary growth* mutants (*max1-1*, *max2-1*, *max2-2*, *max3-9* and *max4-1)* were prepared (as described in Chapter 3) and treated with GR24 as described in Section 4.2.2.

The wild-type calli and calli from the strigolactone biosynthetic mutants *max1* and *max4* responded to GR24 and smoke-water treatments with a significant increase in mass ($P<0.05$) compared with the controls (Table 4.1). Surprisingly, *max3* calli did not respond to GR24 treatments or smoke water treatments (no significant differences observed between the treated and untreated calli). The strigolactone insensitive mutant, *max2-1* unexpectedly demonstrated a significant increase in mass compared with the control. However, as expected, *max2-2* showed no significant differences between the GR24 or smoke treatments and the controls ($P>0.05$). Similar results were observed at both hormone combinations (Table 4.1).

The *max1-1*, *max4-1* and WT calli demonstrated an increased mass compared with the controls ($P<0.05$) following GR24 treatment on AC-free media lacking auxin and cytokinins (Table 4.2), although for *max1-1* calli this only occurred for calli previously sub-cultured on C2 media. No significant differences were observed between the GR24-treated and the control calli of *max2* (both *max2-1* and *max2-2*) and *max3-9* mutants ($P>0.05$) (Table 4.2). No enhanced growth was observed in calli of any of the genotypes on AC-free media in response to treatment with smoke-water.

Table 4.1 Effect of genotype on biomass accumulation following GR24 and smoke-water treatments of *Arabidopsis thaliana* callus in the presence of 2,4-D and kinetin, compared with untreated controls (CTR). (The values are the means± SE).

	*0.5:0.05 mg/L 2,4-D:kinetin			**2:2 mg/L 2,4-D: kinetin		
Genotype	CTR	GR24	Smoke	CTR	GR24	Smoke
max1-1	0.293±0.008^{abc}	0.414±0.05^{ef}	0.423±0.049^{f}	0.499±0.027^{hi}	0.600±0.008^{j}	0.609±0.059^{j}
max2-1	0.279±0.027^{ab}	0.380±0.010^{e}	0.388±0.004^{e}	0.513±0.004^{i}	0.599±0.0057^{j}	0.596±0.0069^{j}
max2-2	0.2970.08±^{bcd}	0.295±0.007^{abcd}	0.292±0.014^{abc}	0.517±0.003^{i}	0.508±0.002^{i}	0.502±0.005^{i}
max3-9	0.310±0.003^{bcd}	0.308±0.007^{bcd}	0.322±0.004^{bc}	0.521±0.015^{i}	0.525±0.005^{i}	0.500±0.006^{i}
max4-1	0.317±0.007^{cd}	0.465±0.004^{gh}	0.445±0.010^{fg}	0.530±0.020^{i}	0.611±0.124^{jk}	0.636±0.006^{k}
Wild-type	0.327±0.006^{bcd}	0.388±0.002^{e}	0.422±0.006^{ef}	0.327±0.005^{d}	0.427±0.005^{f}	0.431±0.003^{fg}

Different letters indicate values that were determined by ANOVA (Fisher LSD test) to be significantly different ($P < 0.05$). * Callus grown at 0.5:0.05 mg/L 2,4-D:kinetin, ** callus grown at 2:2 mg/L 2,4-D:kinetin.

Generally, the increase in mass of the callus that was previously maintained at the high hormone concentration [2:2 mg/L 2,4-D: kinetin] was greater than that of calli previously grown on the low hormone concentration (Tables 4.1 and 4.2). This again emphasizes that the auxin:cytokinin ratio of the sub-culture medium has an influence on the growth and response of callus to GR24.

Table 4.2 Effect of genotype on biomass accumulation following GR24 and smoke-water treatments of *Arabidopsis thaliana* callus in AC-free media, compared with untreated controls (CTR). (The values are the means± SE).

	0.5:0.05 mg/L 2,4-D:kinetin			****2:2 mg/L 2,4-D: kinetin**		
	CTR	GR24	Smoke	CTR	GR24	Smoke
max1-1	0.097±0.0941^{a}	0.136±0.007^{ab}	0.100±0.010^{a}	0.405d±0.043^{ef}	0.502±0.0257 ^{ghi}	0.379±0.021^{e}
max2-1	0.125±0.012^{ab}	0.155±0.0124^{abc}	0.133±0.013^{ab}	0.436±0.021 ^{efg}	0.456±0.026 ^{efgh}	0.401±0.026^{ef}
max2-2	0.0970.009±^{a}	0.099±0.011^{a}	0.102±0.010^{a}	0.519±0.027^{hi}	0.454±0.032^{efghi}	0.477±0.020^{hi}
max3-9	0.197±0.013^{bc}	0.148±0.020^{ab}	0.150±0.013^{ab}	0.449±0.028^{efgh}	0.382±0.024 ^{de}	0.429±0.020^{efg}
max4-1	0.103±0.008^{a}	0.221±0.074^{c}	0.099±0.093^{a}	0.447±0.020^{efgh}	0.526±0.036^{i}	0.430±0.026^{efg}
Wild-type	0.102±0.009^{a}	0.181±0.018^{bc}	0.118±0.007^{ab}	0.189±0.024 ^{bc}	0.342±0.018^{d}	0.215±0.019^{c}

Different letters indicate mean values that were determined by ANOVA (Fisher LSD test) to be significantly different ($P < 0.05$). * Callus that was previously grown at 0.5:0.05 mg/L 2, 4-D: Kinetin, ** callus that was previously grown at 2:2 mg/L 2,4-D: kinetin.

The biomass accumulation shown by *max1-1* and *max4-1* in response to GR24 treatment is to no surprise as these are strigolactone biosynthesis mutants. When the strigolactone biosynthesis mutants are treated with GR24, they revert to the WT phenotype as a result of the exogenously-applied strigolactone (Umehara *et al*., 2008, Gomez-Roldan *et al*., 2008). The results demonstrated by *max3* were not expected, considering the fact that it is also a strigolactone biosynthesis mutant, as it was

assumed that its treatment with GR24 would also result in growth promotion. It has been suggested that several of the available collection of *max* mutants may be leaky (Kohlen *et al*., 2011; Tsuchiya and McCourt, 2012). Additionally, research groups utilising *max* mutants are mostly focussing on *max4* rather than *max3*. Whilst a leaky mutation in the *MAX3* gene would not explain the results observed in this study, it is possible that the mutation results in other, pleiotropic, effects which have not yet been fully characterised. This might also explain the preference for *max4* mutants in the literature.

The response of *max2-1* calli in this study varied. When grown in the presence of both 2,4-D and kinetin, this strigolactone insensitive mutant surprisingly resulted in more biomass than the controls following treatment with GR24 or smoke-water. However, in AC-free media, *max2-1* responded in the expected manner (insensitive to GR24). The inconsistent response of *max2-1* in this study can very probably be attributed to the leakiness of its mutation (Stirnberg *et al*., 2002).

The inability of *max2-2* (and to some extent, *max2-1*) mutants to accumulate extra biomass in response to GR24 and smoke-water suggests that this response is regulated in a MAX2-dependent manner. This discovery widens the known functions of strigolactones and smoke-water and suggests a connection between these two compounds and plant biomass production. However, unlike GR24, smoke-water failed to promote growth in media free of auxins and cytokinins. The differences in responses between smoke-water and GR24 in this study could be due to many factors affecting the growth of callus or the structural differences KAR_1 and GR24. Despite their structural similarities, plant responses to strigolactones and karirikins differ in many ways. For instance, strigolactones are highly effective germination stimulants of the parasitic weeds, whilst karrikins are incapable of doing this (Nelson *et al*., 2009). Although karrikins and strigolactones are both effective light-dependent inhibitors of hypocotyl elongation in *Arabidopsis*, karrikins are more effective in promoting seed germination of Arabidopsis and *Brassica tournefortii* than GR24 (Nelson *et al*., 2009;

2010; Tsuchiya *et al*., 2010). Furthermore, karrikins do not control shoot branching, therefore it is not surprising that differences in response between smoke-water and GR24 were sometimes observed in this study.

4.4 Conclusion

In summary, this work demonstrates that both smoke-water and the synthetic strigolactone analogue GR24 had a positive influence on the growth and biomass yield of *Arabidopsis thaliana* callus. Smoke-water and GR24 elicited a similar response in the presence of exogenously-applied auxin. This also indicates a possibility of a positive cross-talk between smoke-water or GR24 and auxins. Furthermore, the study reveals that cytokinins without an auxin supplement inhibited growth. These findings suggest auxin as a major factor in the growth stimulatory effect taking place in this study. Moreover, this finding clearly reveals that the importance of kinetin in this system could be to enhance the effect of 2,4-D in callus growth. According to the data, it became apparent that both combinations of 2,4-D and kinetin used to grow the callus are suitable to study the responses of *Arabidopsis thaliana* callus to smoke-water and GR24 treatments. Analysis of the growth of callus generated from *A. thaliana* strigolactone biosynthesis and signaling mutants suggests that the growth stimulatory activities of smoke-water and GR24 in this study are MAX2-dependent.

This study also demonstrates a novel role of strigolactones in the promotion of biomass accumulation in callus cultures. The study confirms the broad range of roles played by smoke-water and strigolactones in various developmental processes. Findings of the present study also reveal that strigolactone and smoke-water/KAR_1 actions are not only limited to intact plants and suggest their potential application in agriculture (poverty alleviation and biofuels) and horticulture.

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Chapter 5

Strigolactones promote adventitious root formation in *Arabidopsis thaliana* callus cultures

5.1 Introduction

Unlike animals, plants are faced with distinct constraints of a sessile lifestyle which limits their ability to escape from unfavourable environmental conditions. As a result, they have evolved various strategies to adapt their growth to these adverse conditions (Robert and Friml, 2009). These lifestyle strategies bring about precise developmental responses to certain environmental factors such as light, nutrient supplies, water availability, phototropism, biotic and abiotic stress, which can be perceived by various receptors and signal transduction systems (Teale *et al*., 2006). Different responses permit the adaptation of plants to predictable and exceptional conditions, resulting in regulation and co-ordination of growth and plant architecture (Teale *et al*., 2008; Robert and Friml, 2009).

Roots are simple structures exhibiting an indeterminate growth pattern, which makes them suitable models for studies involving hormones as well as their functions (Teale *et al*., 2008). Development of the root system is crucial for plant life (Takahashi *et* al., 2003; Ludwig-Muller *et al*., 2005). While primary root development from the embryonic stage has received considerable attention, the formation of lateral and adventitious roots is less well understood (Ludwig-Muller, 2005). Lateral and adventitious roots are formed after the embryonic stage. Unlike lateral roots which are initiated from the root pericycle (Benfey and Scheres, 2000; Hardtke, 2006), adventitious roots are formed in non-meristematic tissues after the formation of new meristems (Han *et al*., 2009; De Klerk *et al*., 2001). Adventitious roots (AR) are not easily predictable in their site of origin compared with lateral roots. They may be initiated from the cambium or from

callus via two different pathways, which include direct organogenesis from established cell types or formation from callus tissue, usually following mechanical damage (Dean *et al.,* 2004; Ludwig-Muller *et al.,* 2005).

Root initiation and growth in higher plants is regulated by both external and internal factors; such as temperature, light, sugars and phytohormones (Takahashi *et al.,* 2003; Ruyter-Spira *et al*., 2011). Auxin plays a vital role in the initiation of both the lateral (Malamy and Benfey, 1997; Zhang and Hasenstein, 1999; Guo *et al*., 2005) and adventitious roots (Zimmerman and Wilcoxon, 1935, Sorin *et al*., 2005, Falasca *et al*., 2004). It is mainly required for the process of cell division that either leads to roots or callus (Smith and Thorpe, 1975, Diaz-Sala *et al*., 1996; Greenwood *et al*., 2001). The auxins IAA and IBA were the first phytohormones to be utilized in the stimulation of root cuttings (Ludwig-Muller *et al*., 2005; Cooper, 1936). Currently, auxins are commercially used world-wide to induce rooting in a wide variety of species (Hartman *et al*., 1990; Ludwig-Muller *et al*., 2005).

Polar auxin transport, facilitated by PIN proteins, delivers shoot-derived auxin to the root tip. In the columella root cap, auxin is laterally redistributed towards the epidermal and the cortical cell layers. In the cortical cell layers, a local auxin gradient is established by acropetal auxin transport towards the elongation zone (Blilou *et al*., 2005; Leyser, 2006). This gradient functions in the regulation of cell division and elongation (Ruyter-Spira *et al.,* 2011). Eventually, auxin is driven back to the polar transport stream again. The PIN-dependent auxin recirculation inside the root-tip and the initiation of lateral roots is stimulated by the local accumulation of auxin in the pericycle cells of the root adjacent to xylem vessels (Casimiro *et al*., 2001; Lucas *et al*., 2008; Ruyter-Spira *et al*., 2011).

Several studies have shown cytokinins to be important regulators of adventitious root formation, in addition to auxin (Debnath, 2008; Konieczny *et al*., 2009). Contrary to auxins, cytokinins were demonstrated to negatively regulate adventitious root formation

(Bollmark and Eliasson, 1986; Bollmark *et al*., 1988; De Klerk *et al*., 2001; Clark *et al*., 2004). Although it is well recognized that auxin and cytokinin are key regulators of lateral and adventitious root development, their mechanisms of action are not yet clear. It is possible that other signalling pathways and components play vital roles.

Significant progress has been made in the understanding of the interaction between auxin and strigolactone in the regulation of shoot branching (Ongaro and Leyser, 2008; Hayward *et al*., 2009). In Arabidopsis, *More Axillary Branches 3* and *4* (*MAX3* and *MAX4*) transcripts are positively auxin-regulated in a manner similar to that of orthologous genes in petunia and rice (Foo *et al*., 2005; Snowden *et al*., 2005; Arite *et al*., 2007; Simons *et al*., 2007; Umehara *et al.,* 2008). This finding indicated an evolutionary conservation of this regulation in plants (Hayward *et al*., 2009; Beveridge and Kyozuka, 2010; Koltai *et al*., 2010). The auxin-dependent regulation of *MAX3* and *MAX4* transcripts in plants plays a central role in bud outgrowth control. Hence, large auxin-related reductions in these transcripts correlate with increased axillary branching (Hayward *et al*., 2009). These *MAX3* and *MAX4* transcripts are up-regulated in *max* mutants, indicating a feedback control (Hayward *et al*., 2009). Consistent with this, all *max* mutants have elevated auxin levels in the polar auxin transport stream, suggesting a feedback regulation mediated via auxin signalling (Hayward *et al*., 2009). Thus, both strigolactones and auxin are capable of regulating each other's distribution and levels in a dynamic feedback mechanism (Hayward *et* al., 2009; Mashiguchi *et al*., 2009).

Investigations on the role played by strigolactones in the development of roots has only emerged recently (Koltai *et al*., 2010; Kalpunik *et al*., 2011). Evidence for regulation of root development by strigolactone via the modulation of auxin efflux carriers has been demonstrated in tomato plant (Koltai *et al*., 2010). Upon GR24 treatment, PIN1::GFP, PIN3::GFP and PIN7::GFP green fluorescent protein (GFP) intensities were reduced in the pro-vascular tissue of the primary root tip (Ruyter-Spira *et al*., 2011). In another study conducted under sufficient phosphate (Pi) conditions, application of strigolactones to the roots negatively regulated lateral root (LR) formation (Koltai *et al*., 2011; Ruyter-

Spira *et al.,* 2011). As a result, auxin levels in the leaf tissue were reduced (Ruyter-Spira *et al.,* 2011). When auxin levels were increased by the exogenous application of auxin, GR24 treatment favoured lateral root development. Together these data support the evidence that strigolactones are capable of modulating root architecture.
Most recently, a study by Rasmussen *et al*. (2012) showed a negative regulation of adventitious rooting in Arabidopsis and pea plants. In both species, adventitious root formation was supressed in wild-type and strigolactone biosynthetic mutants, whereas it was enhanced in response mutants, following treatment with GR24. In the same study, analysis of the auxin overproducing line *35S::YUCCA1* revealed an increased number of adventitious roots than the wild-type indicating that auxin plays an important role in adventitious root formation. Treatment with GR24 resulted in a huge reduction of the number of adventitious roots in both the *35S::YUCCA1* mutant and the wild-type, demonstrating a role played by strigolactones regulating root growth.

In another experiment, exogenous application of IAA alone to the wild-type plants promoted the growth of adventitious roots, whereas treatment with GR24 alone greatly decreased the number of adventitious roots (Rasmussen *et al*., 2012). Treatment of the wild-type plants with the combination of IAA and GR24 also reduced the number of adventitious root to the same level as to when it was treated with GR24 only. The difference in the effect of YUCCA1 overexpression and exogenous auxin application could be due to the constitutive or ubiquitous increase in the production of auxin in the *35S::YUCCA1* line, including the rooting zone, in comparison with the exogenous application of auxin (Rasmussen *et al*., 2012).

Furthermore, auxin was able to increase the number of adventitious roots of the *max3* strigolactone biosynthesis mutant. Moreover, the analysis of the *axr1* (auxin response), *max* and *axr1-max* double mutants showed more adventious roots on *max* mutants compared with the wild type (Rasmussen *et al*., 2012). The *axr1* and *axr1-max* double mutants formed almost no roots, supporting a model whereby the *MAX* genes act in an *ARX1*-dependent manner or where *AXR1* is an absolute requirement for adventitious

root formation (Rasmussen *et al*., 2012). These findings highlight the significance of the interaction between strigolactone and auxin in regulating root architecture. These also suggest that this interaction is not only limited to shoot branching.

The first successful root culture method was established in 1934 by Philip White and since then root inducing media has been widely applied with several modifications (Taylor and Van Staden, 1998). Research in root formation is very important within the discipline of plant sciences, especially for horticulture and agronomy where there are economic interests due to the large number of plant species that are difficult to root (Ludwig-Muller *et al.,* 2005).

During the biomass accumulation trials in this study (Chapter 4), GR24 unexpectedly promoted the formation of adventitious roots when the callus was transferred to the AC-free (auxin and cytokinin-free) media. Since this appears counter-intuitive to previous reports in the literature, this interesting phenomenon prompted further investigation into the rhizogenic phenotype shown by the callus in response to GR24 treatment.

5.2 Materials and Methods

5.2.1. Plant material and growth conditions

Calli of the *Arabidopsis thaliana* Columbia-O wild type, *max1-1*, *max2-1*, *max2-2*, *max3-9* and *max4-1* mutant lines were used as plant material (Chapter 3, section 3.2.1). Approximately 50 mg of callus that was previously grown on MS media containing either 2:2 mg/L or 0.5:0.05 mg/L 2,4-D:kinetin was weighed and plated out on auxin- and cytokinin-free (AC-free) medium. The AC-free medium consisted of full MS salts (Murashige and Skoog), 3% sucrose and was supplemented with $1X10^{-7}$ M GR24 or 1:1000 smoke solutions. Thus the media used were identical to those used for sub-culturing (see Chapter 3), except for the exclusion of the auxin (2,4-D) and cytokinin (Kinetin). Five calli were carefully weighed and plated per 90 x 10 mm diameter growth

plate (Greiner Bio-One, Cellstar®), each replicate consisted of 5 plates (n=25). The growth promoting substances were added to the media before adjusting the pH to 5.8 with KOH. Gelrite (2.2 g/L) was then added to the media prior to autoclaving at 121°C, 100 kPa for 20 minutes. The growth plates were incubated in the dark at 23±2°C for 28 days. After four weeks, three of the calli were carefully removed from the plates and gently blotted dry before weighing each callus mass. Calli were then dried in an oven at 70°C for three days to determine the dry mass. All experiments were independently repeated at least three times. Relative growth rate was calculated from the fresh mass, whereby mean mass increase was determined. The remaining two pieces of callus were preserved by quick-freezing in liquid nitrogen, followed by storage at -80°C until further analysis.

5.2.2 Statistical Analysis

Experimental data were analysed using STATISTICA Version 8 (StatSoft Inc. 2005) package. Where applicable, the factorial analysis of variance (ANOVA) to infer differences between treatments was carried out. All percentage data was arcsine transformed to normalize the data before carrying out the ANOVA at the 95% confidence level. When the ANOVA indicated existence of a significant difference between samples, Fisher's LSD Test was carried out as a *post-hoc* test. The return of a *P*-value < 0.05 was regarded as significant.

5.2.3 Molecular analysis of the rhizogenic callus

5.2.3.1 cDNA synthesis and PCR amplification of *MAX* genes in wild-type *Arabidopsis thaliana* callus

In order to test for the expression of the various *MAX* genes in wild-type *Arabidopsis thaliana* callus, reverse transcriptase polymerase chain reaction (RT-PCR) was performed. Primer sets designed to specifically amplify each of the *MAX* genes (*MAX1*, *MAX2*, *MAX3* and *MAX4*) were used. The sequences of all four primer pairs are

provided in Table 5.1. Total RNA was isolated from wild-type callus or inflorescence stem (for comparison purposes). To remove any contaminating DNA, the total RNA was purified using the RNase-free DNase I kit (Fermentas) and RiboLock™ RNase-inhibitor I (Fermentas), according to the manufacturer's specifications. The isolated RNA was quantified with a ND-1000 spectrophotometer (Nanodrop Technologies) and integrity and size range of the total RNA was assessed through gel electrophoresis. First strand cDNA was synthesized using the RevertAid™ HMinusFirst Strand CDNA synthesis kit (Fermentas) and the supplied Oligo $(dT)_{18}$ primers for mRNA-specific amplification with total RNA as template, according to manufacturer's specifications. The cDNA was quantified and PCR reactions were carried out in 20 µL volume containing 20 ng cDNA templates. The optimal PCR reagents are indicated in Table 5. 2, where 4 µL of 5X GoTaq® PCR buffer, 0.4 µL of 10 mM dNTPs (Fermentas), 0.4 µL of 10 mM of forward and reverse primers (Table 5.3) and 0.2 µL of 5U/µLGoTaq® Flexi DNA polymerase (Promega) constituted the PCR reaction mix. The optimal PCR cycling conditions are illustrated in Table 5.4. A negative control reaction was carried out with each PCR reaction either by replacing the primers with de-ionized water or by using non-reverse transcribed total RNA to confirm the absence of contamination. In addition, primers which amplify the constitutively expressed *cyclophlin* gene were used as a positive control. A GeneAMP® PCR system 9700 thermocycler (APPLIED BIOSYSTEMS, USA) was used to conduct all PCR reactions. The PCR products were separated by agarose gel (1% m/v)) electrophoresis in 0.5x TBE buffer. The agarose gels contained 10 mg/mL ethidium bromide (EtBr). The PCR products were visualized on a UV transluminator (APPLIED BIOSYSTEMS, USA) and the sizes of all *MAX* genes were confirmed by comparison against a λ *Pst*I molecular marker, which was generated through the digestion of lambda DNA with the restriction enzyme *Pst*I. The expected sizes of the PCR products for different primers are illustrated in Table 5.5.

Table 5.1 Primer sequences for the amplification of *MAX* genes of *Arabidopsis thaliana* callus.

Primer name	Sequence	Product size
MAX1	F- 5'-GGCCTCTTCTTCACCAGGGACAAGA-3' R- 5'-CAGGCTCGTGGACCGATACCG-3'	1022 bp
MAX2	F- 5'-GCGGCGAGGCTTACACGGTT-3' R- 5'-GCTCGGCTGGCCTGCGTTTA-3'	1078 bp
MAX3	F- 5'-ATCCAATAGGTTCCATAGCGGCT-3' R- 5'-TCCCGACGAGGCAGCAGAGT-3'	521 bp
MAX4	F- 5'-CCGGAGAAGTCGTGGCTAGCG-3' R- 5'-CATGGGTTGCACCGGGTCGA-3'	492 bp
Cyclophilin	F- 5'-TCTTCCTCTTCGGAGCCATA-3' R- 5'-AAGCTGGGAATGATTCGATG-3'	250 bp

#All primers were designed using NCBI/Primer3 BLAST. Primers were synthesizedby Inqaba Biotec TM (SA).

Table 5.2 The concentrations of reagents used for PCR amplification of *MAX* genes of *Arabidopsis thaliana* callus.

Component	Volume	Stock concentration	Final concentration
GoTaq® Flexi buffer	4 µL	5 X	1X
$MgCl_2$,	1.2 µL	25 mM	1.5 mM
dNTP's	0.4 µL	10 mM	0.2 mM
Primer mix (F&R)	0.4 µL	10 mM	0.2 mM
cDNA	0.5 µL	0.547 µg/ µL	0.027 µg /uL
GoTaq® Flexi DNA polymerase	0.2 µL	5 U/µL	0.05 U/ uL
*dH_2O	13.3 µL		
*Total	20 µL		

* Distilled water was added to make up to the final volume of 20 µL

Table 5.3 PCR cycle regime for amplification of *MAX* genes.

PCR step	**Cycling conditions**
Initial denature	94 ℃ – 3 min
Denature	94 ℃ – 30 s
Annealing (Ta)	60 ℃ – 35 s
Extension	72 ℃ – 1 min 30s
Number of cycles	**30**
Final elongation	72 ℃ – 7 min

5.2.3.2 Microarray analysis and processing

The microarray analysis was performed using 4 week old *max4-1* and wild-type calli to compare gene expression profiles in response to GR24, in order to determine how exogenously-applied strigolactones would modulate gene expression. Total RNA was isolated from both GR24-treated and untreated calli of the wild-type Arabidopsis Columbia-O, as well as mutants *max4-1* and *max2-2* mutants (with the same Columbia-O background). The RNA was isolated using the Qiagen RNeasy® Mini Kit, according to manufacturer's instructions. The RNA quantity was measured with a ND-1000 spectrophotometer. Furthermore, RNA degradation was checked by separating the total RNA through gel electrophoresis. Total RNA was precipitated by adding 2.5 volumes of 75% ethanol and 0.1 volume of 3M sodium acetate, prior to courier delivery to the Department of Biology, Friedrich-Alexander-University Erlangen-Nuremberg, Germany where the microarray analysis was performed. The precipitated RNA was purified with an RNeasy Mini Spin Columns (QIAGEN, Valencia, CA; www.qiagen.com). Purified RNA was checked for integrity using an Agilent 2100 Bioanalyzer (vB.02.03 BS1307) according to the manufacturer's instructions (Agilent RNA 6000 Nano Assay Protocol2). Synthesis of cDNA was performed as described in the one-colour microarray-based gene expression analysis protocol provided by Agilent including the one-color RNA spike-in kit (v5.0.1, 2006; Agilent Technologies, Santa Clara, www.agilent.com).

Microarray analyses were performed using Arabidopsis Gene Chips following a gene expression analysis protocol specified by Agilent (v5.0.1, 2006; Agilent Technologies, Santa Clara, www.agilent.com). A CYC3-labelled sample was loaded on the array and hybridized overnight (65℃ for 17h). Samples were washed according to the manufacturer's instruction and scanned on the Agilent Microarray Scanner with extended dynamic range (XDR) at high resolution (5 µm). Data sets were extracted by using the feature extraction software (v9.5.3.1/Agilent Technologies) using a standard protocol. Data analysis was carried out with the use of feature extraction software (v9.5.3.1/Agilent Technologies) according to the manufacturer's instruction. Each hybridized Agilent array was normalized and analysed using the embedded R program in GeneSpringGX (Version 10.0, Agilent Technologies Inc., and Santa Clara, California, USA). Scaling and global normalization were achieved by normalization to the median of all probe sets for all arrays with the use of MAS5.0 algorithm and absolute calls for each probe sets were attained using a lower critical cut-off value of 0.05 and higher critical values of 0.065. Gene selections based on t-test were performed to filter the genes whose residuals between the compared sample pairs were significantly higher than the measured noise level and regarded as differentially expressed. The residual *P*-value obtained by the t-test was corrected for multiple testing by Hierarchical clustering using Pearson cantered, average linkage algorithm with a false discovery rate (FDR) corrected *P*-values ($P < 0.05$) and an associated 2.0 fold change cut-off value in at least one of the gene pairs normalized to the control treatment were regarded as significant.

Gene annotation based on the *Arabidopsis* accession number and/or oligosequence information yielded only cursory information in NCBI BLAST search. Consequently, the *Arabidopsis* accession number and probed oligosequences, respectively, were analyzed in Blast2GO (www.blast2go.de) according to the method described by Conesa *et al*. (2005).

5.2.3.3 Fold change data value adjustments

The original microarray publications judge gene differential expression with regards to the fold change (FC). Usually, 2-fold is considered as traditional cut-off value (McCarthy and Smyth, 2009). Initially, microarray data analysis in this study was conducted based on this criterion, which resulted in considerable complexity and biological noise, such that no precise gene ontology or biochemical pathways could be concluded. Nonetheless, considering the expression values of transcripts based on the data, it was noticed that some meaningful analysis could be performed by adjusting the normal cut-off FC value (2 and 4) to 1.5 and 10. This was due to the fact that this combination criterion found a number of biologically meaningful sets of genes compared with the traditional FC cut-off combination. Based on these criteria and specifications, comparative analysis between the differentially expressed transcripts of untreated *max4-1* and WT was performed. Another comparison was made between the GR24-treated and untreated *max4-1* transcripts.

5.3 Results and Discussion

5.3.1 Strigolactone treatment promotes adventitious rooting in *Arabidopsis thaliana* callus

Upon transfer of the callus onto the auxin- and cytokinin free (AC-free) medium supplemented with GR24, biomass accumulation (Chapter 4) and, interestingly, rhizogenesis occurred. The strigolactone-induced rhizogenic effect was unambiguously enhanced (Fig. 5.1) on the GR24-treated callus which was previously grown at the lower hormone concentration (0.5:0.05 mg/L 2,4-D:kinetin) and then transferred into the AC-Free medium supplemented with GR24, compared with calli previously grown on the higher concentration of hormones (2:2 mg/L 2,4-D:kinetin). As a result, all studies on the root culture were performed on the 0.5:0.05 mg/L 2, 4-D:kinetin medium.

The growth patterns of the rhizogenic calli were similar, characterized by elongated adventitious root (AR) proliferations, which mostly appeared towards the periphery of the callus. The surface of both treated and untreated calli also produced fine hairs.

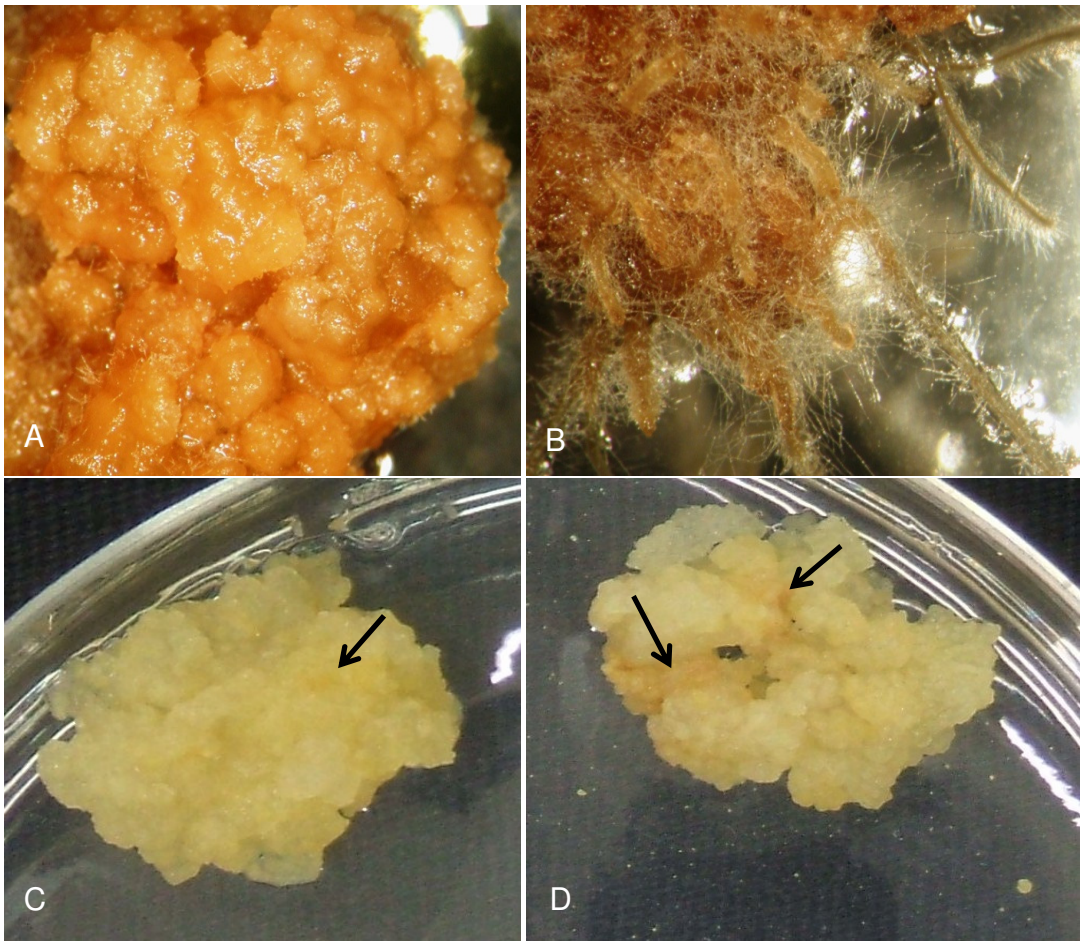

Figure 5.1 Development of adventitious roots and hair-like structures on *Arabidopsis thaliana* wild-type callus. (A) and (B) show callus that was previously grown on media containing 0.5: 0.05 mg/L 2,4D:kinetin following transfer to AC–free MS medium, either with or without GR24 supplementation. (A) Control callus with few hair-like structures; (B) four week old, GR24-treated callus showing adventitious roots with extensive development of the hair-like structures. (C) and (D) show wild-type callus which was previously sub-cultured on media containing 2:2 mg/L 2,4-D:kinetin following transfer to AC–free MS medium in the absence (C) or presence of GR24 (D). These calli do not show any signs of adventitious rooting. Arrows indicate locations where the colour of the callus is beginning to change.

This was not easily noticed in the untreated calli but was particularly pronounced in the GR24-treated callus, where the hairs were also considerably longer (Fig. 5.1). The untreated calli, GR24–treated calli previously grown at high hormone concentrations (2: 2 mg/L 2,4-D:Kinetin), as well as smoke water-treated calli, produced either no, or extremely short adventitious roots. Regardless of the treatment used, the colour of the callus gradually changed (approximately during week two) from cream to dark brown, suggesting some secondary metabolic activity producing phenolic compounds (Fig 5.2).

5.3.2 The effect of strigolactone on adventitious root formation is MAX2 dependent

To assess whether the adventitious root stimulatory effect of strigolactone was dependent on MAX2 function, *Arabidopsis more-axillary growth* mutants (*max1-1*, *max2-1*, *max2-2*, *max3-9* and *max4-1)* were prepared (as described in Chapter 3) and treated with GR24 as described in Section 5.2.1. Rhizogenesis was heavily dependent on the source of the callus, being far more prominent on calli which had previously been grown on the lower hormone concentration than on calli previously grown under high auxin and cytokinins conditions. Of the latter, almost no rooting was observed except for a few replicates in the WT and *max4* mutant calli (approximately 3 out of 25) which produced a small number of roots and hairs (data not shown).

Callus that was previously grown at the lower hormone concentration (0.5: 0.05 mg/L 2,4-D: kinetin) showed varying degrees of rhizogenesis, depending on the genotype treated (Fig 5.2). Strigolactone treatment of the WT resulted in the formation of adventitious roots (AR) and fine hair-like structures, as described in Section 5.3.2. As expected, strigolactone treatment of the strigolactone deficient mutant *max4* also promoted the formation of AR and the fine hairs, in a similar manner to that observed in GR24-treated WT-calli (Figure 5.2). In contrast, the strigolactone signalling mutant *max2-2* did not show AR formation in response to treatment with GR24 and the growth of the fine hair-like structures was reduced. Unexpectedly, *max2-1* demonstrated an

intermediate phenotype (between *max2-2* and WT); approximately half of the GR24-treated callus performed similarly to the *max2-2* callus, whilst the other half responded similarly to the control callus and produced AR and numerous hairs (Figure 5.3. Surprisingly, both the GR24-treated and untreated calli of the *max1-1* and *max3-9* mutants exhibited AR and a more severe phenotype of the fine hairs on their surfaces (Figure 5.3).

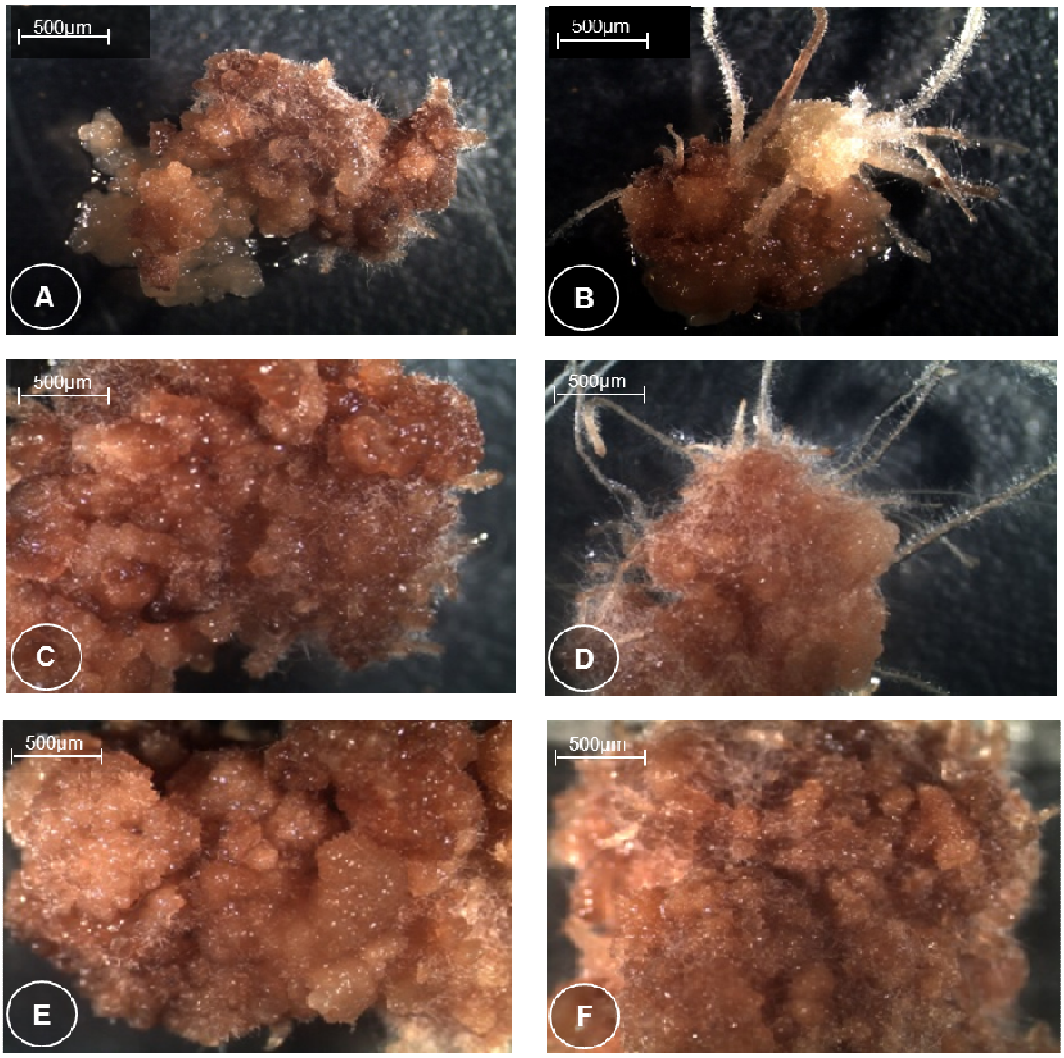

Figure 5.2 Adventitious root formation on *Arabidopsis thaliana* callus cultures in the absence (A,C and E) or presence of GR24 (B,D and F). Top: wild-type (Col-O); middle: *max4-1* mutant, bottom: *max2-2* mutant. Scale bars = 500µm.

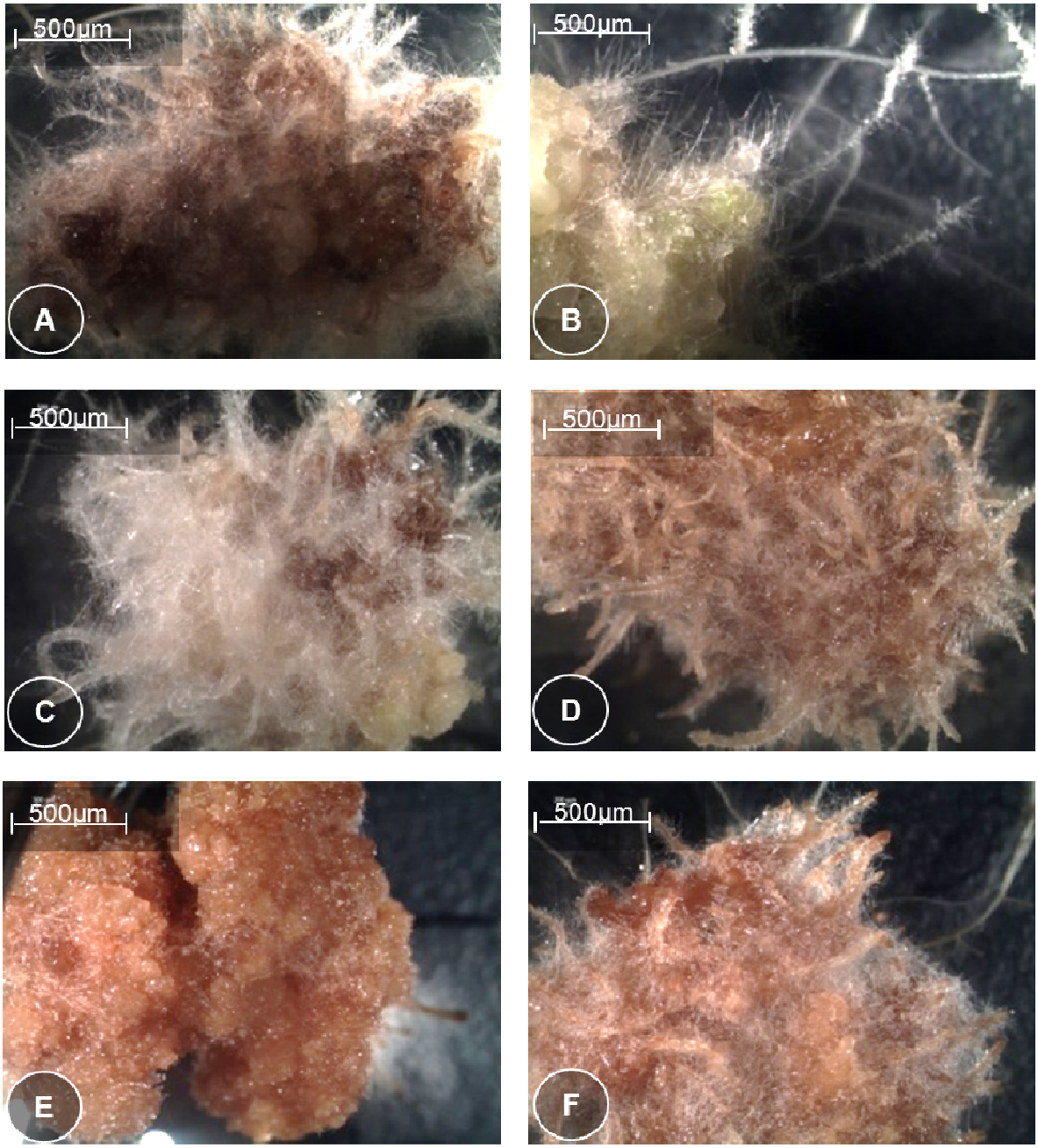

Figure 5.3 Adventitious root formation on *Arabidopsis thaliana* callus cultures in the absence (A,C and E) or presence of GR24 (B,D and F). Top: *max3-9* mutant, middle: *max1-1* mutant, bottom: *max2-1* mutant. Scale bars = 500µm.

Evidence for regulation of root development by strigolactones is emerging (Koltai *et al*., 2010; Kalpunik *et al*., 2010; Ruyter-Spira *et al*., 2011). In this study, a possible role for strigolactones in promoting adventitious root formation has been discovered. Evidence for this role is based on the analyses of *Arabidopsis* strigolactone biosynthesis or signalling mutants and application of GR24, the synthetic strigolactone analog. A summary of the responses of calli (previously grown under low hormone conditions) from the various genotypes is given in Table 5.4. Differences on the degree of adventitious root formation between the WT calli and strigolactone-deficient calli provide evidence for the role of strigolactones in regulating adventitious root formation.

Table 5.4 Summary of the effects of the synthetic strigolactone (GR24) on *Arabidopsis thaliana* WT and *max*-mutant calli.

Genotype	-GR24	+ GR24	Hair-like structures	Biomass accumulation
Wt Columbia-O	NR	R	++	Yes
max3-9 (CCD7)	RR	RR	+++	No
max4-1 (CCD8)	NR	R	++	Yes
max1-1 (Cytochrome P450)	RR	RR	++	No
max2-1 (F-box)	N/R	N/R	+++	Yes/No
max2-2 (F-box)	NR	NR	+	No

NR = No roots, R = Roots, RR = more roots, N/R = Intermediate.

+ = few; ++ = numerous; +++ = extremely numerous.

The lack of sensitivity of *max2-2* in response to GR24, together with the sensitivity of *max4-1* to this strigolactone analog suggests that the effect of GR24 on adventitious rooting is mediated in a *MAX2*-dependent manner. However, the responses of the other mutants are somewhat confounding. The inconsistent response of *max2-1* in this study could be attributed to the leakiness of its mutation, which means that it may or may not always respond in the expected fashion (Stirnberg *et al*., 2002). An experiment to check the *MAX2* gene expression level in the *max2-2* and *max2-1* mutants to verify this hypothesis was carried out. This experiment was, however, not successful (data not included), as both mutants produce full length mRNA transcripts that are indistinguishable from the wild-type transcript without sequence analysis. Other means to test the expression of the *MAX2* gene in these mutants should be employed in future. Although information on this would have been invaluable for future understanding; this experiment could not be conducted due to time constraints.

The responses shown by the *max3* and *max1* mutants were unexpected. As both mutants are impaired in strigolactone biosynthesis, it was speculated that these two mutants would respond in a similar fashion to *max4* and the WT callus. In previous studies, treatment with synthetic strigolactone (GR24) or grafting onto WT-rootstock experiments reverted all three of the strigolactone biosynthesis mutants to WT phenotype (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). The reason why callus of the *max3* mutant showed an unexpected response in both biomass accumulation and root formation is at the moment not understood. However, it may be due to its leakiness or pleiotropic effects as described in Chapter 4.

A study by Kohlen *et al.* (2011) revealed that *max1* mutant has compromised strigolactone levels, it also acts in more downstream biosynthesis steps and has an ability to alter the strigolactone analog (GR24) and thereby increase its biological activity (Ruyter-Spira *et al*., 2011). This finding could suggest a reason why *max1* showed unexpected results in this study. Previously, authors have reported that low strigolactone concentrations inhibit lateral root initiation via the MAX2 response pathway

(Kalpunik *et al*., 2011, Ruyter-Spira *et al*., 2011). In addition to lateral root inhibition, it was reported that strigolactones further enhanced root-hair elongation (Kalpunik *et al*., 2011). Together all these data support a role of strigolactones in root developmental processes.

Callus that was previously grown at the high hormone concentration (2:2 mg/L 2,4-D:Kinetin) in this study was not efficient in producing roots or the hair-like structures. There is evidence from other studies that strigolactones enhance rooting when auxin levels are low, but inhibit rhizogenesis in the presence of high auxin levels. Kalpunik *et al*. (2011) observed that excess GR24 disturbs auxin efflux, resulting in high cellular concentrations of auxin, which consequently leads to a reduction in the GR24-induced root-hair elongation. It is thus possible that in this study, for callus initially cultured under high hormone concentrations (2:2 mg/L 2, 4-D: Kinetin), an excess of auxin remained in the callus which resulted in an inhibition of the root response in the presence of GR24, whilst the same was not true for the calli initially cultured under the low hormone conditions (0.5:0.05 mg/L 2,4-D: kinetin). Hence it has been documented that exogenous application of auxin affects the process of root formation in a concentration-dependent manner (Koltai *et al*., 2011).

Ruyter-Spira *et al*. (2011) postulated that the major role of strigolactones in the development of plants lies in the coordinated balanced control of the shoot architecture ratio under the ever-changing environments. Mencuccini (2003) reported that *in vitro* rooting depends on many exogenous and endogenous factors including the genetic origin, physiological influences, environment (temperature and light) and constituents of the nutrient medium. Thus the varying growth responses of the different genotypes in a variety of MS media could be ascribed to genetic variation, nutrient medium or even temperature. The varying responses between smoke-water and GR24 in this study could be due to the above-mentioned reasons or their structural differences, as it has been reported that these two compounds sometimes have distinct roles (modes of action) or species specificity (Nelson *et al*., 2010).

Findings in this study are in contrast with the results of Rasmussen *et al.* (2012). A possible explanation for this may be due to differences in the concentration of GR24. It is well documented that phytohormones generally influence plants in a concentration dependent manner. For instance part of the study of Rasmussen *et al.* (2012) involved concentration-response experiments whereby it was discovered that GR24 was only effective in supressing adventitious rooting in Arabidopsis at a concentration of 100 nM and higher. On the other hand, the *rms5* mutant of pea and the wild type started to respond to concentrations of 1nM and 100 nM and higher respectively. Despite the abilities of these two plants to respond to a variety of GR24 concentrations, 1000 nM was selected as the best concentration for Arabidopsis, whereas 500 nM was preferred for the pea plants. Similarly, earlier-on in this study (Chapter 4), dose-response experiments using smoke-water were performed, whereby a dilution of 1000 resulted in best growth promotion. Another possible explanation for this could be the fact that different plant material and media were used. Rasmussen *et al.* (2012) used whole plants, whereas the current study used callus cultures. The differences in results may also be associated with differences in growth media.

5.3.3 Total RNA isolation

To unravel the molecular mechanisms underlying the promotion of AR by the strigolactone treatment, WT, *max4-1* and *max2-2* calli were selected for further analysis. Several different methods for total RNA isolation were tested in order to isolate good quality RNA for genetic expression studies. These included a CTAB-based method (White *et al*., 2008) and a phenol-based method. These initial methods used for isolation of RNA in this study failed either due to low yield or degradation of the RNA. RNA degradation is often attributed to an increase in the RNases or RNA degrading compounds in the tissues (White *et al*., 2008). The low RNA yield may have been due to phenolic compounds, as it was noticed that the rhizogenic calli gradually changed colour from cream to brown, indicating some production of polyphenols, as described above. Polysaccharides, polyphenols and secondary metabolites are some of the major elements restricting yield and quality of RNA in a number of species and some tissues (Logemann *et al*., 1987; White *et al*., 2008). Polyphenols usually bind to nucleic acids

and form higher molecular weight complexes, which makes it difficult to successfully extract RNA of high yield and quality (Moyo *et al*., 2008). The polysaccharides often co-precipitate with nucleic acids and interfere with quantification.

Eventually, a Guanadine-HCl based method (Logeman *et al*., 1987) and the Qiagen RNeasy® Mini Kit produced intact RNA (Fig. 5.4 and Fig. 5.5 respectively). However, the concentration of RNA extracted using the latter method was higher than that achieved using the Guanadine-HCl method (Table 5.5). The Qiagen RNeasy® Mini Kit was therefore chosen as the best method to obtain sufficient RNA for further downstream analysis.

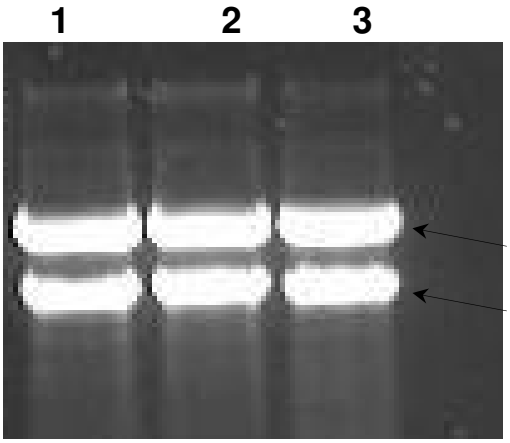

Figure 5.4 RNA isolated from callus of *max2-2* (lane1), *max4-1* (lane 2) and wild-type (lane 3) using the Qiagen.

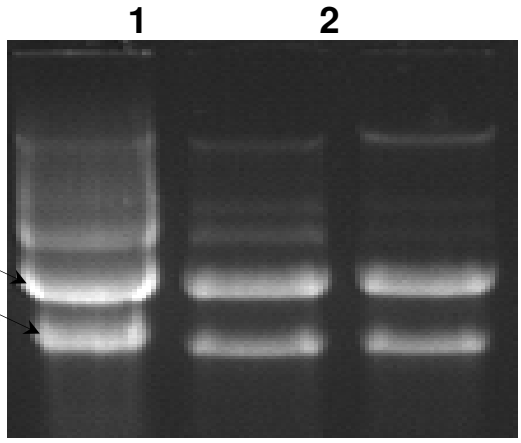

Figure 5.5 RNA isolated from callus of *max2-2* (lane 1), *max4-1* (lane 2) and wild-type (lane 4) using the guanidine-HCl method (Logeman *et*

Table 5.5 Concentration of total RNA from Arabidopsis wild-type Col-O and *max* mutants (*max2-2* and *max4-1*)

	Guanadine-HCl	Qiagen RNeasy® Mini Kit
Genotype	RNA (ng μL^{-1})	RNA (ng μL^{-1})
Col-O	335.5	1295.5
max4-1	860.9	1446.2
max2-2	1000	1422.7

5.3.4 Test for expression of *MAX* genes

Since it is unknown from the literature whether any of the *MAX* genes are expressed in callus tissues *in vitro*, RT-PCR was used to test for the expression of these genes in wild-type callus, as well as in the inflorescence stem of *A. thaliana* Columbia-O, where all the *MAX* genes have been reported to be expressed. The PCR analysis resulted in successful amplification of 1022 bp (*MAX1*), 1078 bp (*MAX2*), 521 bp (*MAX3*) and 492 bp (*MAX4*) products in both calli and stem. Expression of all of the *MAX* genes could be detected in both tissues (Figure 5.6).

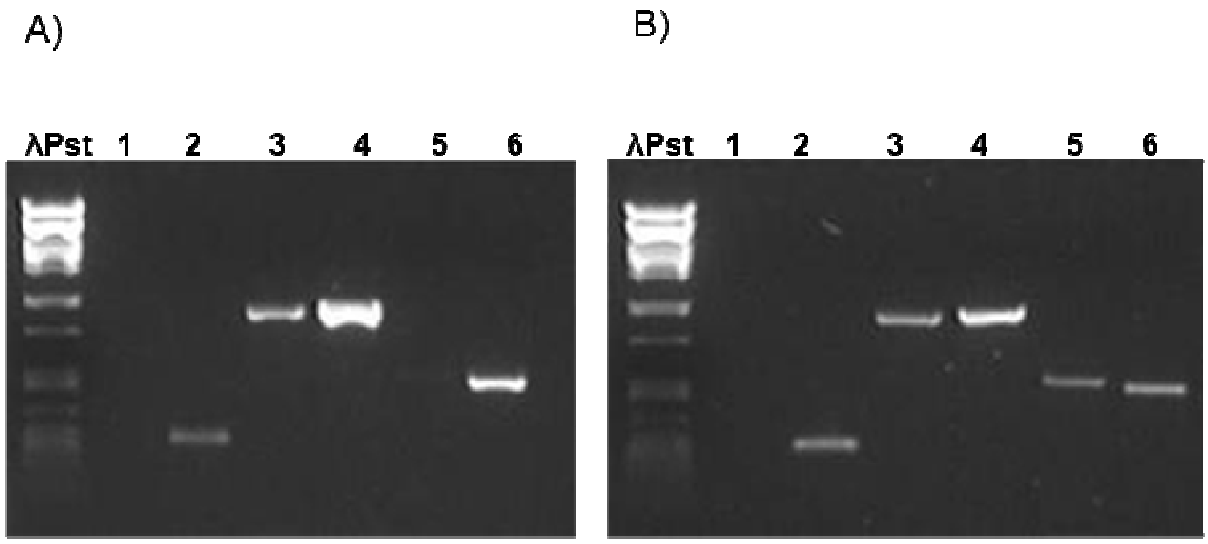

Figure 5.6 Expression of *MAX* genes in callus (A) and inflorescence stem (B) of *Arabidopsis thaliana* Columbia-O using RT-PCR. Lane1-negative (water) control; Lane 2- Positive control (*cyclophyllin*, 250 bp); Lane 3- *MAX1* (1022 bp); Lane 4-*MAX2* (1078 bp); Lane 5-*MAX3* (521 bp); Lane-*MAX4* (492 bp).

5.3.5 Gene expression profiling

5.3.5.1 *MAX2*-dependent signal transduction

In order to elucidate the molecular changes induced by GR24 on the rhizogenic calli, microarray analysis was performed on strigolactone deficient mutant (*max4-1*) and the wild-type (Col-O) callus. This experiment also included *max2-2*, *max4-1* and the wild type Col-O. Successful RNA isolation was achieved from all three genotypes and was further processed for microarray analysis. However, during the initial steps of microarray experiments, the RNA isolated from *max2-2* was found to have been degraded, presumably during the transportation process to Germany. Despite several attempts, including the use of several fresh batches of RNA, this problem persisted, thus the *max2-2* was not used for further analysis. The reason why it was difficult to perform further downstream analysis on this mutant is still not clear. Additionally, this factor was a severe constraint, as the original experimental design utilised *max2-2* as a negative control for biomass/rhizogenesis related genes, as this mutant is unable to perceive the strigolactone signal. Thus, any genes expressed in this mutant could be excluded from analyses in the WT or *max4* mutant, helping to narrow the search for genes involved in biomass accumulation and rhizogenesis.

5.4.5.2 Gene expression profiling reveals regulation of stress-related transcripts

Firstly, microarray analysis was done between the untreated wild-type and *max4-1* mutant calli. The goal of this experiment was to examine gene expression patterns between the normal growing wild-type and the normal growing *max4-1* mutant calli. In other words, this experiment aimed to look at gene expression changes as a result of endogenous strigolactone levels, since the *max4-1* mutant callus should not be able to generate strigolactones. The analysis indicated 706 genes with varied expression patterns (a full list of these genes is provided in Appendix A). The differentially expressed genes were categorized with respect to function (Gene ontology, The Arabidopsis Information Resource [TAIR] database). Of the expressed genes, 92 (13%) encoded various transcription factors (Figure 5.7). These included ABI3, AP2/EREBP, AS2, bHLH, bZIP, C2C2 zinc finger family, G2-like, MYB and MADS transcription

factors, which are responsible for many plant biological processes such as development, senescence, morphogenesis as well as abiotic and biotic stress.

It was also observed that approximately 50 genes (7%) encoded ubiquitin proteasome associated proteins. Amongst these, 15 genes which were up-regulated belonged to the SCF complex of multi-proteins. Twelve of these genes which encoded subunits of the E3 ubiquitin ligases were down-regulated, followed by an up-regulation of three CULLIN genes. CULLIN is also one of the major components of the SCF complexes. The E3 ubiquitin ligases confer substrate specificity to the ubiquitin proteasome pathway (Moon *et al*., 2004).

Of interest was the up-regulation of genes that encode various transporters such as *MITOGEN-ACTIVATED PROTEIN KINASE* (*MPK12*) and CBL-interacting protein kinases (*CIPK1).* The MPK12 protein regulates a number of physiological processes including responses to phytohormones and environmental stress (Lee *et al*., 2009). The latter protein is also regulated in response to stress (Ok *et al*., 2005).

Other stress-related genes included *RAPETALA* (*RAP2.4*) and *ROOT HAIR DEFECTIVE4* (*RHD4)* which are implicated in drought and salt stress responses. It has been reported that *rap2.4* loss-of-function mutant and constitutive overexpression of RAP4.2 resulted in defects in a number of developmental processes, including drought tolerance (Lin *et al*., 2008). The *RHD4* gene encodes Phosphatidylinositol-4-Phosphate phosphatase, which is usually induced as a result of osmotic stress and is responsible for proper development of root hairs (Bonangelino *et al*., 2002; Thole *et al*., 2008).

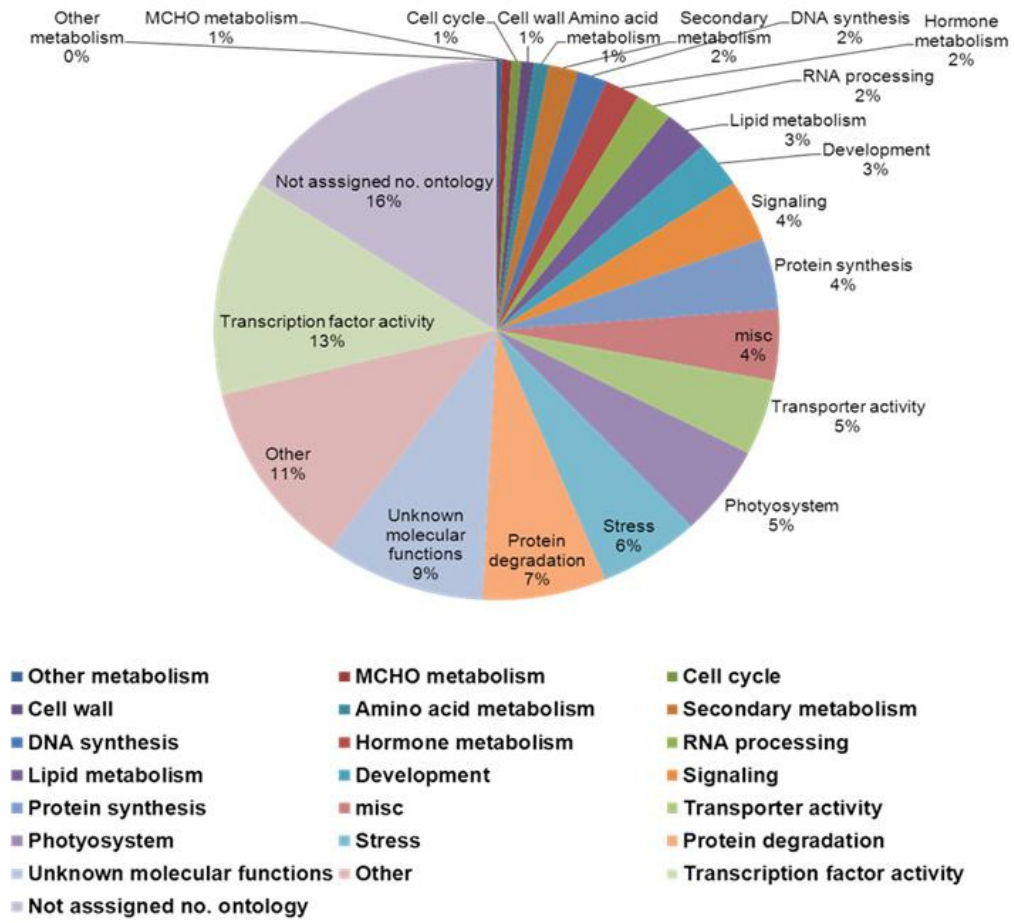

Figure 5.7 Functional grouping of co-expressed genes (up and down-regulated) in untreated WT and *max4* calli. Genes were categorized by function at The Arabidopsis Information Resource (TAIR) databases.

Interestingly, significant changes in expression of genes encoding photosystem (PSI and PSII) subunits were also observed (16-up and 12-down regulated). For instance, *AT5G24930* (B-box zinc finger) has been reported to play an important role in light signalling (Mashiguchi *et al*., 2009). Several Photosystem members are membrane complexes found in the thylakoid membrane of oxygenic photosynthetic organisms. The PSII functions in a series of light-induced electron transfer reactions resulting to splitting of the water molecule into hydrogen and oxygen (Umena *et al*., 2011). Photosystem II functioning is one of the most sensitive indicators of environmental stress in photosynthetic plants. It was surprising to observe the expression of these genes in calli, particularly since the calli were grown in the dark. Furthermore, genes associated with secondary metabolism were also differentially expressed although at very low levels (10 out of 709). Five of these genes were up-regulated, whereas the remaining half was down-regulated. Secondary metabolites play a significant role in the adaptation of plants to the ever-changing environment and to overcome stress constraints (Edreva *et al*., 2007).

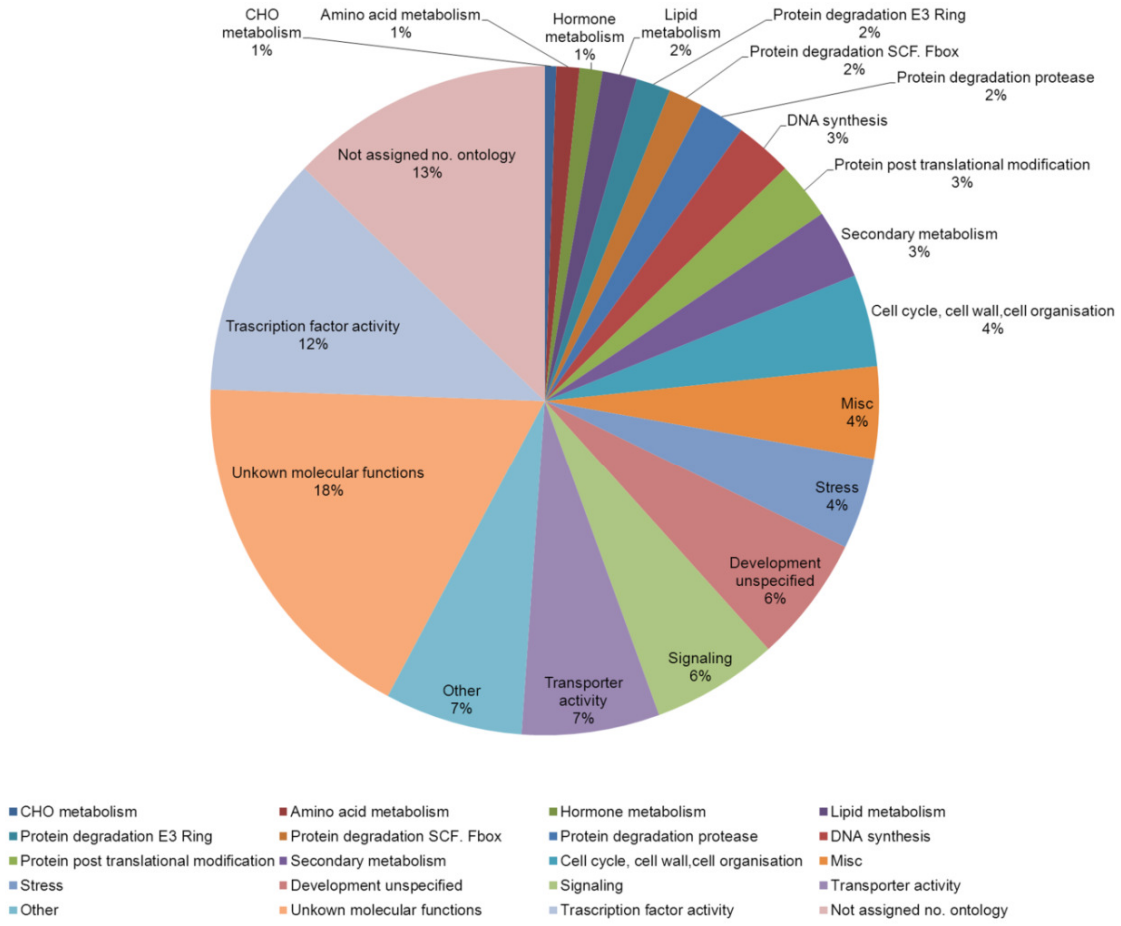

Figure 5.8 Functional grouping of Co-expressed genes (up- and down-regulated) in GR24 treated and untreated *max4-1* calli. Genes by function at The Arabidopsis Information Resource (TAIR) database.

Next, microarray analysis was conducted on GR24-treated and control calli of the strigolactone-deficient mutant *max4-1*. The aim of this experiment was to examine whether exogenous application of strigolactone to calli of this mutant would cause any changes in gene expression. From this analysis, 180 genes demonstrated a strong change in the level of expression (Figure 5.8). The gene data list revealed similarities with the data obtained when comparing the untreated wild-type and *max4-1* (Figure 5.7). This finding was not surprising because *max4-1* is a strigolactone biosynthesis mutant. Experiments showed that strigolactone biosynthesis mutants revert to wild-type following treatment with GR24 (Umehara *et al*., 2008, Gomez-Roldan *et al*., 2008), as described in section 5.3.2.

Thus, the data revealed the involvement of numerous genes involved in transcription factor activity, such as C2H2 zinc finger family, MYB domain transcription factors which are all involved in many plant processes including responses to abiotic and biotic stress (Zhang and Wang, 2005). Again, three members of the SCF complex were up-regulated following GR24 treatment, indicating that rhizogenesis may involve controlled degradation of specific proteins.

Genes encoding proteins involved in signalling such as *CALCINEURIN B-LIKE* were up-regulated in response to GR24. *CALCINEURIN B-LIKE* proteins are important calcium sensors in plant-specific calcium signalling (Kudla *et al*., 1999; Guo *et al*., 2001). Calcium plays an important role as a messenger in mediating various defence responses during environmental stress (Malho, 1998; Snedden and Fromm, 1998).

Some stress related genes such as *ALLENE OXIDE CYCLASE* (*AOC*), *EARLY RESPONSIVE TO DEHYDRATION* (*ERD*) were down-regulated in this system following GR24 treatment. AOC is involved in response to desiccation, water deprivation and is a key enzyme in jasmonic acid biosynthesis. Jasmonic acid (JA) regulates plant responses to biotic and abiotic stress as well as some plant growth and development processes (Hofmann *et al*., 2006). The *ERD* gene is rapidly induced in response to various abiotic and biotic stimuli (Rai *et al*., 2012).

Many of the transport related genes were down-regulated (10) whereas only two were up-regulated in response to GR24 treatment. A gene encoding for the PROLINE TRANSPORTER protein, which is associated with the transport of proline, was among the down-regulated genes. Proline usually accumulates in plants during environmental stress, functioning as an osmoprotectant (Ueda *et al*., 2001).

In this study, microarray data indicated changes in the expression of members of the SCF complex, suggesting an intense involvement of the ubiquitin-mediated protein degradation during the growth of the callus. This result is not surprising because MAX2, which is involved in strigolactone signalling forms part of the SCF complex (Stirnberg *et al*., 2007) as described in Chapter 2. This suggests degradation of as yet unknown proteins by SCF^{MAX2} and other SCF complexes during the growth of the callus. It was previously shown that protein ubiquitination and the subsequent protein degradation are essential for many plant physiological developmental processes including light responses, abiotic and biotic stress in plants (Mazzucotelli *et al.,* 2006; Somers and Fujuwara, 2009). Ubiquitin proteins and their importance in plants have been dealt with in Chapter 2. CULLIN3 (CUL3) proteins, along with BTB-domain proteins, form a complex of Cullin-RING ubiquitin ligases referred to as CRL3s. The CRL3s mediate rapid degradation of major regulatory proteins in eukaryotes. It has been reported that CUL3 is capable of regulating plant growth and development both embryonically and post-embryonically. Additionally, in *Arabidopsis*, CUL3 controls primary root growth and patterning in an ethylene dependent and independent manner (Thomann *et al*., 2009). In accordance with the expression data, the abundance of the members of the SCF complex reflects the functional activity of these proteins during the growth of the callus.

The occurrence of stress-related genes was extensive among the responses (Figure 5.7 and 5.8). From the data, it is possible that rhizogenesis involved the activation of some stress-related genes by GR24, followed by the up-regulation of the light-genes, either sooner or later. In accordance with this, Steenkamp (2011) found a connection between strigolactones and exposure to stress. Under stress conditions, GR24 was able to promote the growth of *N. benthamiana* seedlings. The overall growth of these seedlings was enhanced through the facilitation of lateral root formation and increasing the growth of the above-ground structure in order to compensate for the

increased shoot growth (Steenkamp, 2011). These findings (Figures 5.9 and 5.10) suggest some kind of stress mechanism taking place during the growth of the calli in AC-Free system. Consistently, genes related to hormones which are associated with stress (ABA and ethylene) and defence (jasmonic) acid were up-regulated in response to GR24 (Figures 5.9 and 5.10). Moreover, evidence that suggests a stress mechanism taking place in the AC-free media is that calli growing in this media experienced a gradual change of colour (week two) from cream to dark brown, particularly in the WT and *max4-4* callus lines. These two genotypes also demonstrated a positive biomass accumulation besides root formation in response to GR24 treatment.

However, the data also indicated that some of the expressed transcription factors such as RAP4.2 and B-box zinc finger are not only associated with stress but they are also implicated in light signalling (Mashiguchi *et al*., 2009). Moreover, genes such as those that encode PSI and PSII were differentially up-regulated. This result was interesting, considering the fact that the callus was grown in the dark. Hence the expression of these light associated genes could probably represent an intrinsic response to GR24, rather than a response to light.

Light quality and intensity were demonstrated to affect multiple processes in plants, including determination of shoot architecture and shade avoidance response. Several studies revealed a connection between light and strigolactone pathways (Waldie *et al*., 2010). The seedlings of *max2* were discovered to be hyposensitive to red and far red light. Furthermore, a number of genes such as Rubisco and chlorophyll protein precursors exhibited a slower rate of induction upon exposure to red light in *max2* mutants compared to the wild-type (Shen *et al*., 2007). Because light quality has been shown to suppress shoot branching as part of the shade avoidance response, it is possible that strigolactones are one of the mediators of that response. Additionally, strigolactone mutants in pea plant retained or even enhanced their sensitivity to day length in terms of their shoot branching architecture compared to the wild-type (Franklin *et al*., 2007, Beveridge *et al*., 2003). It has been shown that MAX2 is greatly involved in light signalling pathways at the seedling stage (Stirnberg *et al*., 2002; Shen *et al*., 2007). In Arabidopsis seedlings, the B-box transcription factors were suggested to be candidates for signalling molecules in MAX2-dependent light signalling. The light-signalling genes were induced shortly (90 min) after the

seedlings were exposed to GR24 (Mashiguchi *et al*., 2009). Hence it is tempting to suggest that the expression of these light-associated genes in this study could indicate the MAX2-dependent light signalling during the formation of adventitious roots.

In another study, transcription profiling of GR24-treated wild-type tomato and the *Sl-ORT1* mutant (strigolactone deficient) revealed an expression of genes putatively involved in light harvesting, indicating that strigolactones are positive regulators of light harvesting (Dor *et al*., 2010; Koltai *et al*., 2010; Mayzlish-Gati *et al*., 2010). This was inferred from the list of genes triggered by GR24 treatment, which was enriched in putatively light-associated genes. Interestingly, this was apparent whether auxin was exogenously supplied or not. In accordance with this, in this study, GR24 was able to stimulate rhizogenesis even in the absence of exogenously-applied auxin.

Microarray data showed strong up-regulation of light-associated genes in response to GR24. This is also could be due to the fact that the investigations were carried on callus, lacking chlorophyll because it was grown in the dark and it was expected that expression of the light-associated genes should have been reduced. According to Mayzlish-Gati *et al*. (2010), the *Sl-ORT1* mutant containing less chlorophyll, had demonstrated a reduction in the expression of light harvesting associated genes. From this finding, it was further deduced that in the shoot, strigolactones may control shoot branching in response to light or light harvesting. In this study, it was clear that strigolactones promoted rhizogenesis in *Arabidopsis thaliana* callus even if light was absent (growth took place in the dark room). Thus, it is easy to assume that the growth enhancement (rhizogenesis) taking place in this study somehow depends on light signalling but in a light-independent manner.

However, it is necessary to conduct more research in order to confirm or refute this hypothesis because in several cases, similar growth responses can be induced by distinct signalling pathways. Further investigations are thus necessary to clarify the connection between strigolactone and light in light-regulated process taking place in dark- grown callus. One possible way of doing this would be to apply GR24 to a variety of mutants which are null or defective in light-signalling. These lines include a mutant (*phyA phyB phyD phyE*) defective in red/far-red photoreception, a

cryptochrome double mutant (*cry1 cry2*) defective in blue light photoreception and a transgenic line overexpressing a negative regulator of light signaling, *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*), which reduces sensitivity to light (Franklin *et al*., 2005, Tsuchiya *et al*., 2010). The use of *Sl-ORT1* mutant would also be of value in the elucidation of the interaction between strigolactones and light-signaling on the growth stimulatory effects observed in this study.

Regrettably, the microarray analysis done in this study was not authoratative as there were complications with *max2* and the FC values, thus it is important that this experiment be conducted again in future. Furthermore it would be interesting to also validate the microarray screening with specific assays such as qPCR.

5.5 Conclusion

In this study, GR24 application clearly had a positive rhizogenic effect on *Arabidopsis thaliana* callus. The results indicate that this adventitious rooting is positively regulated by strigolactones via the MAX2 signalling pathway. Root growth is a tightly regulated process, co-ordinately controlled by several phytohormones of which auxin and cytokinins play major roles (Casimiro *et al*., 2001). Hence, although the effects of the endogenous auxin and cytokinin on the strigolactone's rhizogenic action in this study still need to be examined, it is tempting to speculate that strigolactones promote adventitious rooting at low auxin and cytokinin levels. Relevant phytohormone inhibitors may become useful tools in elucidating the interaction between strigolactones, auxins, and cytokinins in this study. In conclusion, the results indicate that strigolactones have an additional hormonal role in plants, suggesting a beneficial use in the propagation of commercially important species.

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Chapter 6

Final conclusions and future prospects

6.1 Introduction

Over the years, *Arabidopsis thaliana* emerged as an important model organism for research in plant physiology, molecular biology and genetics because of a number of traits that make it desirable for research (Lawrence, 1976; Dean, 1993; Meyerowitz and Somerville, 1994; Pyke, 1994; Meinke *et al*., 1998). The advantages of using callus cultures are profound and could significantly contribute to the study of the complex regulatory and signalling pathways responsible for plant growth and development. The main aim of this study was to investigate the plant growth promoting potential of the strigolactone analogue GR24 and smoke-water, in light of their structural similarities, using an *Arabidopsis* cell culture model system as an alternative to a whole plant study system. The main concepts developed during the course of this study will be highlighted in this chapter.

6.2 Induction of high quality *Arabidopsis thaliana* callus

In order to provide a platform for studying the plant growth promoting abilities of smoke-water and GR24, callus of good quality and quantity had to be produced not only from the wild-type *Arabidopsis thaliana* Col-O callus, but also from five *max* mutants i.e, *max1-1, max2-1, max2-2, max3-9 and max4-1*. All genotypes showed successful callus induction in both hormone combinations used (2:2 2,4-D: kinetin and 0.5:0.05 2,4-D:Kinetin). The protocol developed here is thus simple, efficient and reproducible.

6.3 Strigolactones demonstrated dual effects on the growth of *Arabidopsis thaliana* callus

On the basis of the data produced in this study, the synthetic strigolactone and smoke-water have both demonstrated the ability to promote the biomass of

Arabidopsis thaliana callus in a statistically significant manner. Additionally, GR24 not only promoted biomass accumulation, but stimulated adventitious root formation as well. These findings may prove valuable for future tissue culture research. Additionally, they have created an important platform for further investigations into adventitious root formation in plants.

These observations are in accordance with the results of Kotze (2010), whereby GR24 was able to promote the accumulation of biomass in *Nicotiana benthamiana* seedlings. Furthermore, this study supports the recent emerging information about the regulation of root growth by strigolactones. This study also aimed at comparing the growth of the calli grown at different concentrations of 2,4-D and kinetin in response to GR24 and smoke-water treatments. From the data, it was apparent that callus that was previously grown at the lower hormone concentration (0.5:0.05 mg/L 2,4-D:kinetin) and then treated with smoke-water or GR24 produced more biomass than the callus which was previously grown at the high hormone concentration. Additionally, callus that was previously grown at a low hormone concentration and then treated with GR24 showed a much greater ability to produce roots compared with callus grown at the high hormone concentration. Thus, although a dual positive role of GR24 in the promotion of *Arabidopsis thaliana* callus growth was demonstrated, the concentration of the hormones used to grow the callus affects its response to GR24. From this data, it was concluded that although callus grown at both hormone concentrations managed to accumulate greater biomass in response to GR24 treatment than the control callus, the low hormone concentration is the best concentration to consider for further analysis and future investigations. Findings in this study, contribute to literature on plant growth promoting abilities of smoke-water and GR24.

6.4 Strigolactone affects *Arabidopsis thaliana* callus growth in a MAX2-dependent manner

In this study, the mediation of the callus growth and adventitious rooting processes via MAX2 could not be definitely explained at a molecular level through microarray analysis. Nonetheless, mutant studies strongly suggest that the biomass accumulation and rooting are mediated through MAX2. This was made apparent by

the fact that both biomass accumulation and adventitious rooting were observed in *max4-1* and the wild-type, but not in the strigolactone insensitive *max2-2* callus. However, further research is necessary to confirm this concept at molecular level. Phytohormone profiling would have brought more knowledge and understanding on any regulation in hormone homeostasis and possible cross talk taking place during the growth promotion processes in this study. However, due to time constraints and limited resources these investigations could not be conducted.

6.5 Smoke-water promotes biomass accumulation but not rhizogenesis

This study has also demonstrated the ability of smoke in promoting the biomass of *Arabidopsis* callus. As with the strigolactones, this appears to occur in a MAX2-dependent manner. Interestingly, in the presence of auxin and cytokinin, the biomass promoting ability of smoke-water was even more than that of the GR24. This indicated the smoke-water's strong plant growth promoting potential. However, unlike GR24, smoke-water treatment in AC-free medium was not able to promote rhizogenesis. This observation was attributed to the many external and internal factors that affect *in vitro* rooting or the structural differences between GR24 and karrikins. The dissimilar responses demonstrated by smoke to that of GR24 in this study are not surprising because these two plant growth regulators have previously been shown to have common as well as distinct responses, as explained in Chapter 4.

6.6 Conclusion

Even though the exact mechanisms by which GR24 and smoke-water regulate growth in this system could not be concluded, there were some valuable points noted for future investigations. For instance, although gene expression profiling generated a considerable amount of biological noise, there were indications that rhizogenesis may be linked to the activation of one or more stress mechanisms. It would be valuable in future to investigate this phenomenon, as a strigolactone involvement in the enhancement of the ability of plants to tolerate stress has been suggested by Steenkamp (2011), as described in Chapter 5.

In conclusion, smoke and GR24 application in terms of use in horticulture and agriculture is still novel and requires more research. These two compounds have potential benefits in that they can be both used at very low concentrations. Smoke in particular, has been shown to be having non- toxic effects and may reduce microbial infections on crop plants (Kulkarni *et al*., 2011). This is one aspect that must still be investigated when using GR24. The growth stimulatory effects of both smoke and GR24 in plants under stress or normal conditions may help increase crop productivity. In addition, the use of these compounds may be affordable and cost-effective method of improving crops for resource poor farmers especially in developing countries. However, more research is still needed on this subject in order to fully understand the full potential of this method of utilizing smoke and GR24 as a tool for improving agricultural yields and horticultural systems, in addition to understanding their molecular pathways.

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Appendices

Appendix A: Summary of the statistical data for the callus growth experiments.

Table A1 A summary of the statistical results (univariate test) after ANOVA for the growth of the calli (5 mg (W1) and 50 mg (W2) on different concentrations of auxin and cytokinin.

	SS	Degrees of freedom	MS	*F* test	*P*-value
Intercept	283854.0	1	283854.0	10278.79	0.000000
Concentration	67.6	1	67.6	2.45	0.120583
Weight (g)	8101.0	1	8101.0	293.35	0.000000
Treatment	32718.3	1	32718.3	1184.78	0.000000
Concentration)*Weight (g)	0.7	1	0.7	0.02	0.878294
Concentration)*Treatment	96.6	1	96.6	3.50	0.064109
Weight(g)*Treatment	188.7	1	188.7	6.83	0.010180
Concentration*weight(g)*Treatment	271.4	1	271.4	9.83	0.002197
Error		112		-	-

Table A. 2.1 Mass increase (fresh weight) results correlating interactions related to various treatments (Control: CTR, GR24 and smoke) grown on MS medium containing 2:2 mg/L 2,4-D:kinetin and 0.5:0.05 mg/L 2,4-D:kinetin.

	SS	Degrees of freedom	MS	*F* test	*P*-value
Intercept	100.4079	1	100.4079	10278.79	0.000000
Concentration	0.9308	1	0.9308	2.45	0.000000
Treatment	2.4659	2	1.2330	1184.78	0.000000
Concentration)*Treatment	0.0610	2	0.0305	3.50	0.010436
Error	0.9327	144	0.0065	-	-

Table A. 2.2 Dry mass results correlating interactions related to various treatments (Control, GR24 and smoke) grown on MS medium containing 2:2 mg/L 2,4-D:kinetin and 0.5:0.05 mg/L 2,4-D:kinetin.

	SS	Degrees of freedom	MS	*F* test	*P*-value
Intercept	0.396294	1	0.396294	2193.509	0.000000
Concentration	0.035964	2	0.017982	99.531	0.000000
Treatment	0.012881	1	0.012881	71.295	0.000000
Concentration*Treatment	0.000745	2	0.000373	2.063	0.130846
Error	0.026016	144	0.000181	-	-

Table A.3 Investigation of the correlation between different treatments in *Arabidopsis thaliana* calli grown on MS medium with either 2,4-D or kinetin only, supplemented with GR24 or smoke.

	SS	Degrees of freedom	MS	*F* test	*P*-value
Intercept	148.5974	1	148.5974	6781.546	0.000000
Concentration	0.4329	1	0.4329	19.756	0.000013
Treatment	29.2590	5	5.8518	267.059	0.000000
Concentration*Treatment	4.6029	5	0.9206	42.0313	0.000000
Error	6.3107	288	0.00219	-	-

Table A.4.1 Fresh weight results correlating interactions related to various treatments (Control, GR24 and smoke) grown on AC-free MS medium.

	SS	Degrees of freedom	MS	*F* test	*P*-value
Intercept	25.00716	1	25.00716	1394.419	0.000000
Concentration	0.80772	1	0.80772	45.039	0.000013
Treatment	4.18042	2	2.09021	116.552	0.000000
Concentration*Treatment	0.32420	2	0.16210	9.039	0.0002000
Error	2.58246	144	0.01793	-	-

Table A. 4.2 Dry mass results correlating interactions related to various treatments (Control, GR24 and smoke) grown on AC-free MS medium.

	SS	Degrees of freedom	MS	*F* test	*P*-value
Intercept	0.2530775	1	0.2530775	1186.161	0.000000
Concentration	0.0349101	2	0.0349101	81.811	0.000013
Treatment	0.0624118	1	0.0624118	292.51	0.000000
Concentration*Treatment	1.78249	2	0.16210	2.061	0.0002000
Error	3.07236	144	0.01793	-	-

Appendix B: Microarray analysis

Table B1 706 genes differentially expressed between the strigolactone biosynthesis mutant, *max4-1,* and wild type calli.

Array ID	Gene symbol	Fold change	Gene name
A_84_P86021	ATHSFA6B	10.89271	DNA binding
A_84_P86287	AT1G49230	10.611601	Zinc finger (c3hc4-type ring finger)
A_84_P86290	AT1G49320	10.549577	BURP domain containing protein
A_P70557	AT3G06220	10.460859	DNA binding
A_P84889	KCS10	10.33252	Acyltransferase
A_P86269	AT4G10820	10.225527	F-box family protein
A_P80780	SBPASE	10.190696	SBPASE (sedoheptulose-bisphosphatase)
A_P72056	ATGSTU14	9.842294	Glutathione transferase
A_P85196	PSBTN	9.638597	PSBTN (photosystem ii subunit t)
A_P80696	AT5G61750	9.625686	Cupin family protein
A_P85719	AT4G15248	9.578169	Zinc ion binding
A_P86340	AT5G37870	9.467257	Seven in absentia (sina) family protein
A_P85561	-	9.397478	Rep: s-adenosylmethionine decarboxylase
A_P80572	AT4G03156	9.343351	Small gtpase-related
A_P85676	ATPC1	9.069656	ATPC1; enzyme regulator
A_P83222	-	8.707614	Rep: f3f9.8 - (mouse-ear cress)
A_P82140	THI1	8.691748	THI1; protein homodimerization
A_P85173	MYB22	8.669294	MYB22 (myb domain protein 22)
A_P54910	AT5G25470	8.372765	DNA binding
A_P85677	AT4G38970	8.184213	Fructose-bisphosphate aldolase
A_P84818	AT4G39770	7.8875685	Trehalose-6-phosphate phosphatase
A_P80480	AT1G55260	7.8156857	Lipid binding
A_P804831	THIC	7.6169906	Thic (thiaminc)
A_P853531	AT1G47610	7.574111	Transducin family protein
A_P808174	scpl23	7.565404	SCPL23 (serine carboxypeptidase-like 23)
A_P770302	HAF2	7.5533724	HAF2 (histone acetyltransferase)

A_P795434	anac011	7.4806504	ANAC011 (arabidopsis nac domain)
A_P795285	AT5G24050	6.981317	DNA binding
A_P799845	GUN4	6.9604692	GUN4; enzyme binding
A_P787005	AT3G43580	6.7271957	Beta-galactosidase
A_P802368	ANAC026	6.5800014	ANAC026; transcription factor
A_P793735	CAB3	6.4661226	CAB3 (chlorophyll a/b binding)
A_P793391	-	6.407671	Rep: photosystem i reaction center subunit ii
A_P801290	AT3G20640	6.1464744	Ethylene-responsive protein -related
A_P789767	-	6.042978	AT2G05300 mrna sequence
A_P812448	ANAC019	4.7418075	Flowers and buds
A_P789482	PSAD1	4.339232	PSAD-1 (photosystem i subunit d-1)
A_P802642	AT3G01350	3.9399636	Proton-dependent oligopeptide transport
A_P867676	-	3.3502913	Rep: copper/zinc superoxide dismutase precursor
A_P806178	PSBP1	3.105917	PSBP-1 (photosystem ii)
A_P807342	AT5G66010	3.0131254	RNA binding
A_P807361	AT4G38700	2.9892325	Disease resistance
A_P808087	AT4G39070	2.9356096	Zinc finger (b-box type) family protein
A_P806931	AtGolS1	2.9037151	ATGOLS1 (galactinol synthase 1); transferase
A_P804905	AT1G49750	2.887768	Leucine-rich repeat family protein
A_P822692	HSP70T2	2.6013484	HSP70T-2 (heat-shock protein 70t-2)
A_84_P785579	AT5G03560	2.4702654	Nucleobase:cation symporter
A_84_P787799	AT1G29730	2.297516	ATP binding
A_84_P795958	GRP3S	2.1985638	GRP3S (glycine-rich protein 3)
A_84_P862854	LINC1	1.9839723	LINC1 (little nuclei1)
A_84_P857154	AtbZIP52	1.9332169	ATBZIP52 (basic leucine zipper 52)
A_84_P847260	PDS1	1.9088888	PDS1 (phytoene desaturation)
A_84_P799713	PAB4	1.9034404	PAB4 (poly(a) binding protein 4)
A_84_P786969	AT3G21580	1.9022064	Cobalt ion transmembrane transporter
A_84_P802675	AT1G63290	1.7919694	Ribulose-phosphate 3-epimerase
A_84_P754253	AT1G31070	1.7571992	UDP-n-acetylglucosamine pyrophosphorylase-related
A_84_P730299	AT3G53230	1.7191939	Cell division cycle protein
A_84_P811265	AT4G39960	1.6434956	Full-length cdna complete sequence

A_84_P827422	ATPHB2	1.6009215	Atphb2 (prohibitin 2)
A_84_P800063	AT1G68300	1.5059192	Universal stress protein (usp) family protein
A_84_P801706	ERD15	1.5044386	Erd15 (early responsive to dehydration 15)
A_84_P801073	XIK	1.503005	Xik; motor/ protein binding
A_84_P795460	CCMH	1.5019486	CCMH; oxidoreductase
A_84_P728206	AT1G04790	1.5014532	Zinc finger (c3hc4-type ring finger) family protein
A_84_P803322	ATMAK3	1.50023	ATMAK3; n-acetyltransferase
A_84_P548604	RGXT1	9.07701	RGXT1 (rhamnogalacturonan xylosyltransferase 1)
A_84_P15205	AT5G21170	1.6423333	5'-amp-activated protein kinase beta-2 subunit, putative
A_84_P829246	AT1G75340	1.7181495	Zinc finger (ccch-type) family protein
A_84_P14962	FRA8	1.615751	Fiber 8); glucuronosyltransferase
A_84_P127161	AG	3.8676276	AG (agamous)
A_84_P825382	-	3.8228257	Rep: photosystem i reaction center
A_84_P542081	AT5G48570	3.7947154	Peptidyl-prolyl cis-trans isomerase
A_84_P20840	AT4G33150	1.7055324	Lysine-ketoglutarate reductase
A_84_P307040	AT3G29270	1.5838919	Ubiquitin-protein ligase
A_84_P21961	AT4G00160	2.688855	F-box family protein
A_84_P21207	AT5G29000	2.6666617	MYB family transcription factor
A_84_P11687	MAF5	2.8569336	MAF5 (mads affecting flowering 5)
A_84_P21935	PSAG	2.840658	PSAG (photosystem i subunit g)
A_84_P824135	AT1G17520	1.658763	DNA-binding protein, putative
A_84_P12313	AT1G64110	3.4939568	AAA-type atpase family protein
A_84_P830085	AT4G16680	2.2339869	RNA helicase, putative
A_84_P21787	AOS	2.1006005	AOS (allene oxide synthase)
A_84_P14462	CAB1	5.957229	CAB1 (chlorophyll a/b binding protein 1)
A_84_P827163	HMT3	5.93243	HMT3; homocysteine s-methyltransferase
A_84_P67034	AT4G11320	5.896448	Cysteine proteinase, putative
A_84_P785093	BAM2	5.7862277	BAM2 (beta-amylase 2)
A_84_P20900	AT5G58160	2.445365	Actin binding
A_84_P21992	MPK12	2.4446163	MPK12 (mitogen-activated protein kinase 12)
A_84_P842914	AT4G35270	1.7266738	RWP-RK domain-containing protein
A_84_P22123	CIPK1	1.6153747	CIPK1 (cbl-interacting protein kinase 1)

A_84_P501599	PSBR	2.508842	PSBR (photosystem ii subunit r)
A_84_P13175	PLPB	1.722977	PLPB (pas/lov protein b)
A_84_P169313	CSP41A	2.7427177	CSP41A binding / poly(u) binding
A_84_P11579	OBE2	1.638812	OBE2 (oberon2); protein binding
A_84_P843146	AT1G12290	1.6379379	Disease resistance protein
A_84_P831967	-	1.6369083	None
A_84_P21738	AT5G64580	1.5280784	AAA-type atpase family protein
A_84_P212638	GTB1	2.057307	GTB1; RNA binding / hydrolase
A_84_P18474	AT1G73530	2.024185	RNA recognition motif (rrm)-containing protein
A_84_P23194	GAMMA-VPE	1.7336736	GAMMA-VPE (gamma vacuolar processing)
A_84_P761052	AT4G28320	1.5399238	Glycosyl hydrolase family 5 protein
A_84_P23174	ATBAG3	1.539616	ATBAG3 (bcl-2-associated athanogene 3)
A_84_P18877	PRF4	2.2871032	PRF4 (profilin 4); actin binding
A_84_P860159	PSAG	4.2645793	PSAG (photosystem i subunit g)
A_84_P16384	HDG10	3.6272428	HDG10 (homeodomain glabrous 10)
A_84_P16982	PSBR	2.5389066	PSBR (photosystem ii subunit r)
A_84_P21463	DOT4	1.7691445	DOT4 (defectively organized tributaries 4)
A_84_P13064	ATDAD1	1.7654325	ATDAD1 (defender against apoptotic death 1)
A_84_P830543	AT3G04450	1.76442	Transcription factor
A_84_P831991	AT1G62130	1.761433	AAA-type atpase family protein
A_84_P23777	AT3G02750	1.7601331	PP2C family protein
A_84_P20555	AtATG18h	1.7587738	ATATG18H
A_84_P752961	NF-YA5	4.0550365	NF-YA5 (nuclear factor y, subunit a5)
A_84_P20646	PSBO1	3.4290652	PSBO1 (ps ii oxygen-evolving complex 1)
A_84_P538753	DDB2	3.427633	DDB2 (damaged dna-binding 2)
A_84_P828910	TZP	3.427293	TZP; dna binding
A_84_P257040	AT1G65250	3.4230354	Protein kinase family protein
A_84_P15572	SULTR3;1	3.415047	SULTR3;1 (sulfate transporter 3;1)
A_84_P16277	AT2G32430	1.9840096	Galactosyltransferase family protein
A_84_P787962	TTR1	1.910635	TTR1; protein binding / transcription factor
A_84_P825937	AT1G02080	1.7623295	Transcriptional regulator-related
A_84_P750936	AT1G48390	1.757654	Syntaxin-related family protein

A_84_P15322	P40	1.6172525	P40; structural constituent of ribosome
A_84_P167773	AT1G72960	4.4896584	Root hair defective 3 gtp-binding family protein
A_84_P21662	SDG37	1.738553	SDG37; zinc ion binding
A_84_P800991	AT5G45280	1.7291099	Pectinacetylesterase, putative
A_84_P703748	-	7.8823357	-
A_84_P23679	RPB5C	2.118071	RPB5C (rna polymerase ii fifth largest subunit, c)
A_84_P810368	TIFY10B	2.0729809	TIFY10B
A_84_P15450	AT1G52510	2.0723736	Hydrolase, alpha/beta fold family protein
A_84_P23544	ALDH11A3	2.0722914	ALDH11A3; 3-chloroallyl aldehyde dehydrogenase
A_84_P534953	AT1G32530	2.042084	Zinc finger (c3hc4-type ring finger) family protein
A_84_P23463	AT5G38850	1.7830766	Disease resistance protein (tir-nbs-lrr class), putative
A_84_P147008	RAP2.4	1.7824644	RAP2.4 (related to ap2 4); dna binding / transcription factor
A_84_P854259	XBAT32	1.7329987	XBAT32; protein binding / zinc ion binding
A_84_P859300	AT3G56210	1.6333594	Binding
A_84_P798820	AT5G19150	1.8321418	Carbohydrate kinase family
A_84_P810987	ABF1	1.8315595	ABF1 (abscisic acid responsive element-binding factor 1)
A_84_P810995	AT2G16485	1.8295316	DNA binding / nucleic acid binding / protein binding
A_84_P20353	CNGC18	1.7505866	CNGC18 (cyclic nucleotide-gated channel 18)
A_84_P786499	UGT75B2	1.5840654	UGT75B2 (udp-glucosyl transferase 75b2)
A_84_P209738	AT2G03820	1.6932468	Nonsense-mediated mrna decay nmd3 family protein
A_84_P824725	AT3G15890	1.6918528	Protein kinase family protein
A_84_P12778	AT1G80440	2.7943375	Kelch repeat-containing f-box family protein
A_84_P18994	AT5G05340	2.1768816	Peroxidase, putative
A_84_P16183	AT4G33150	2.796337	Lysine-ketoglutarate reductase/saccharopine dehydrogenase
A_84_P17415	AT5G05400	2.7925344	Disease resistance protein (cc-nbs-lrr class)
A_84_P12052	AT3G29760	1.8183268	NLI interacting factor (nif) family protein
A_84_P12178	AT5G04540	2.1523085	Phosphatase/ protein tyrosine phosphatase
A_84_P15310	ERD15	1.6443412	ERD15 (early responsive to dehydration 15)
A_84_P17844	ZIP11	2.1898532	ZIP11 (zinc transporter 11 precursor)
A_84_P266040	GAMMA-VPE	2.1863549	Gamma-vpe (gamma vacuolar processing enzyme)
A_84_P16530	AtPP2-B6	2.653308	ATPP2-b6 (phloem protein 2-b6); carbohydrate binding
A_84_P796465	AT4G13840	2.634244	Transferase family protein

A_84_P22892	AT4G13230	2.2851384	Late embryogenesis abundant domain-containing protein
A_84_P140529	ATOCT3	3.7747288	ATOCT3 (organic cation/carnitine transporter 3)
A_84_P162983	AT2G25240	2.6984177	Serine-type endopeptidase inhibitor
A_84_P19066	AT1G63400	1.6249197	Pentatricopeptide (ppr) repeat-containing protein
A_84_P788429	AT3G58490	1.5954127	Phosphatidic acid phosphatase family protein / pap2 family protein
A_84_P795337	CAB3	7.2261605	CAB3 (chlorophyll a/b binding protein 3); chlorophyll binding
A_84_P550233	AT3G54050	3.7696507	Fructose-1,6-bisphosphatase, putative
A_84_P206798	GAPA	3.3609853	GAPA (glyceraldehyde 3-phosphate dehydrogenase)
A_84_P834200	AT4G01230	2.0240602	Reticulon family protein (rtnlb7)
A_84_P19223	-	1.6613605	-
A_84_P18511	-	1.7310383	-
A_84_P843889	WRKY35	1.7092661	WRKY35 (wrky dna-binding protein 35); transcription factor
A_84_P209378	AT5G37740	1.7083997	C2 domain-containing protein
A_84_P825181	OPR1	1.7047282	OPR1; 12-oxophytodienoate reductase
A_84_P805666	-	1.6100316	-
A_84_P709830	MSH6	6.829858	MSH6 (muts homolog 6); damaged dna binding
A_84_P17662	GAMMA-VPE	1.703837	Gamma-VPE (gamma vacuolar processing enzyme)
A_84_P18353	AT5G16860	1.6143315	Pentatricopeptide (ppr) repeat-containing protein
A_84_P16053	AT4G19870	1.511713	Kelch repeat-containing f-box family protein
A_84_P829914	ALDH7B4	1.5114912	ALDH7b4 (aldehyde dehydrogenase 7b4)
A_84_P56690	NTT	4.7947245	NTT (no transmitting tract)
A_84_P19613	AT3G09240	2.5392883	Protein kinase-related
A_84_P12409	AT5G61400	2.0636094	Pentatricopeptide (ppr) repeat-containing protein
A_84_P858074	AT3G19480	2.6984034	D-3-phosphoglycerate dehydrogenase, putative / 3-pgdh, putative
A_84_P17857	SAM-2	2.2115643	SAM-2 (s-adenosylmethionine synthetase 2)
A_84_P812891	ATMRP12	2.1252909	ATMRP12; atpase, coupled to transmembrane -
A_84_P15460	ATRPAC43	1.5543928	ATRPAC43; DNA binding
A_84_P18394	TIF3C2	4.0977855	TIF3C2; translation initiation factor
A_84_P10297	PUB23	3.4067519	PUB23 (plant u-box 23); ubiquitin-protein ligase
A_84_P565889	AOC1	3.3526785	AOC1 (allene oxide cyclase 1); allene-oxide cyclase
A_84_P20821	-	1.6630493	-
A_84_P521218	AT2G36350	3.3313885	Protein kinase, putative

A_84_P820808	GAPA	3.3234491	GAPA (glyceraldehyde 3-phosphate dehydrogenas)
A_84_P849768	MKK9	3.2469544	MKK9 (map kinase kinase 9)
A_84_P831373	AT3G23210	2.146013	Basic helix-loop-helix (bhlh) family protein
A_84_P22255	AT3G58980	4.8238616	F-box family protein
A_84_P15394	LHCA4	4.809552	LHCA4 (light-harvesting chlorophyll-protein complex
A_84_P845392	AT1G52700	5.5458255	Phospholipase/carboxylesterase family protein
A_84_P815150	CLE21	5.001274	CLE21 (clavata3/esr-related 21); protein binding / receptor binding
A_84_P110642	AT3G48030	5.000352	Hypoxia-responsive family protein / zinc finger family protein
A_84_P861588	AT3G54400	4.9667473	Aspartyl protease family protein
A_84_P21647	AT2G30220	1.7497085	GDSL-motif lipase/hydrolase family protein
A_84_P837504	AT5G01760	1.7496966	VHS domain-containing protein / gat domain-containing protein
A_84_P10926	ATTIM23-2	1.7358547	ATTIM23-2; p-p-bond-hydrolysis-driven protein transmembrane
A_84_P815131	AT5G27750	1.7355266	Transporter
A_84_P12781	AT5G18610	1.5973922	F-box family protein
A_84_P217118	AT4G29020	4.075721	Protein kinase family protein
A_84_P808742	-	1.983932	Glycine-rich protein
A_84_P13325	-	4.617076	-
A_84_P21716	CAB1	3.512451	CAB1 (chlorophyll a/b binding protein 1); chlorophyll binding
A_84_P834383	PSBP-1	3.5075622	PSBP-1 (photosystem ii subunit p-1); poly(u) binding
A_84_P852243	HCF107	5.725823	HCF107 (high chlorophyll fluorescent 107); binding
A_84_P830157	AT3G47290	4.8808203	Phosphoinositide-specific phospholipase c family protein
A_84_P836098	ABI4	4.164302	ABI4 (aba insensitive 4); dna binding / transcription factor
A_84_P815049	AT5G19900	2.4570134	Prli-interacting factor, putative
A_84_P89769	AGL31	3.0540314	AGL31 (agamous like mads-box protein 31); transcription factor
A_84_P11052	AT3G58200	2.06373	Meprin and traf homology domain-containing protein
A_84_P533507	PSAL	3.4571512	PSAL (photosystem i subunit l)
A_84_P21648	AT2G24280	2.3409662	Serine carboxypeptidase s28 family protein
A_84_P13808	ATMRP10	1.7807593	ATMRP10; atpase, coupled to transmembrane
A_84_P831342	AT5G38410	9.331415	Ribulose bisphosphate carboxylase small chain
A_84_P12951	AT5G37950	2.3574672	Transferase, transferring hexosyl groups
A_84_P18744	AT1G66610	2.3531642	Seven in absentia (sina) protein, putative
A_84_P12141	AT2G36370	2.3457634	Ubiquitin-protein ligase

A_84_P825225	AT4G03440	2.2244542	Ankyrin repeat family protein
A_84_P868545	AT3G01570	2.2219288	Glycine-rich protein / oleosin
A_84_P23567	PDE149	2.220312	PDE149 (pigment defective 149)
A_84_P607453	AT1G74270	1.5707223	60s ribosomal protein l35a (rpl35ac)
A_84_P22621	AT1G69400	1.5702511	Transducin family protein / wd-40 repeat family protein
A_84_P846242	HSP18.2	1.5695381	HSP18.2 (heat shock protein 18.2)
A_84_P14424	AT5G53320	1.5694593	Leucine-rich repeat transmembrane protein kinase, putative
A_84_P863621	CIPK1	1.5681461	CIPK1 (cbl-interacting protein kinase 1); kinase/ protein binding
A_84_P14683	-	1.5674852	-
A_84_P23879	HMGB5	1.6278934	HMGB5 (high mobility group b 5)
A_84_P23171	AT5G35100	1.626523	Constituent of chromatin / transcription factor
A_84_P15503	AT2G16210	1.6262374	Peptidyl-prolyl cis-trans isomerase
A_84_P12713	AT1G31180	2.0254579	Transcriptional factor b3 family protein
A_84_P596337	PSAD-1	4.8042727	3-isopropylmalate dehydrogenase, chloroplast, putative
A_84_P15294	AT3G23410	2.0405436	PSAD-1 (photosystem i subunit d-1)
A_84_P226439	FAMT	2.0381353	Alcohol oxidase-related
A_84_P837120	GLT1	2.031277	FAMT (farnesoic acid carboxyl-o-methyltransferase)
A_84_P22430	AT5G46250	1.6818956	GLT1; glutamate synthase (nadh)
A_84_P830222	UBP12	2.017315	RNA recognition motif (rrm)-containing protein
A_84_P98716	ASML2	4.132029	UBP12 (ubiquitin-specific protease 12)
A_84_P788621	AT1G21100	4.0726156	ASML2 (activator of spomin::luc2); transcription activator
A_84_P11163	LTP5	2.3362713	O-methyltransferase, putative
A_84_P767975	AT3G26480	2.3282332	lTP5 (lipid transfer protein 5); lipid transporter
A_84_P821739	CPK23	2.3200984	CPK23; atp binding / calcium ion binding
A_84_P609853	AT4G21850	2.124807	Methionine sulfoxide reductase
A_84_P832965	-	1.7774644	-
A_84_P796670	MT1C	1.7972238	MT1C; copper ion binding
A_84_P10469	AT5G53490	2.372098	Thylakoid lumenal 17.4 kda protein, chloroplast
A_84_P199724	RTFL3	4.282819	RTFL3 (rotundifolia like 3)
A_84_P21306	AT1G48400	2.4470603	F-box family protein
A_84_P860855	-	1.6674938	-
A_84_P15620	HDG12	2.4867852	HDG12 (homeodomain glabrous 12); transcription factor

A_84_P822525	AtNMNAT	1.5627999	Atnmnat (a. thaliana nicotinate/nicotinamide/adenyltransferase)
A_84_P849473	AT1G21630	1.5603957	Calcium-binding ef hand family protein
A_84_P811636	AT3G30770	5.0962186	ATP binding / aminoacyl-trna ligase/ nucleotide binding
A_84_P10205	ATHSP90.1	5.074542	ATHSP90.1 (heat shock protein 90.1); atp binding
A_84_P845822	PRK	5.046579	CSR1 (chlorsulfuron/imidazolinone resistant 1)
A_84_P16735	CAB1	5.018393	SULTR3;1 (sulfate transporter 3;1)
A_84_P703544	CAB1	6.8167653	ATBAG7 (bcl-2-associated athanogene 7)
A_84_P20567	ACC2	1.6121643	EMB2739 (embryo defective 2739)
A_84_P20336	AT4G00290	1.6115649	CBL10 (calcineurin b-like 10); calcium ion binding
A_84_P825125	ATBAG3	1.611477	T-complex protein 11
A_84_P584493	AT4G01050	3.1808248	Serine-rich protein-related
A_84_P21584	AT2G47420	1.6748705	Phospholipase c
A_84_P528334	AT5G05100	1.673267	AAA-type atpase family protein
A_84_P51800	AT2G27580	1.6725128	RPP5 (recognition of peronospora parasitica 5)
A_84_P838825	CSR1	1.6716114	ATGLR3.3; intracellular ligand-gated ion channel
A_84_P788534	SULTR3;1	4.1337934	20s proteasome alpha subunit b, putative
A_84_P10100	ATBAG7	1.7796011	Proton-dependent oligopeptide transport
A_84_P562958	emb2739	1.7795233	EMB2739 (embryo defective 2739)
A_84_P13379	CBL10	2.0999932	CBL10 (calcineurin b-like 10); calcium ion binding
A_84_P506433	AT1G22930	2.1713364	T-complex protein 11
A_84_P818463	AT5G25280	2.161641	Serine-rich protein-related
A_84_P277340	AT3G16020	2.1575198	Phospholipase C
A_84_P806704	AT1G64110	3.1051688	AAA-type atpase family protein
A_84_P847047	RPP5	3.0849242	RPP5 (recognition of peronospora parasitica 5)
A_84_P23870	GLR3.3	1.5886248	ATGLR3.3; intracellular ligand-gated ion channel
A_84_P23876	AT1G79210	1.5349684	20s Proteasome alpha subunit b, putative
A_84_P14770	AT1G59740	4.530596	Proton-dependent oligopeptide transport family protein
A_84_P832201	AT5G12270	3.793559	Oxidoreductase, 2og-fe(ii) oxygenase family protein
A_84_P23861	RPP1	1.5937952	RPP1(recognition of peronospora parasitica 1)
A_84_P23370	-	3.3043897	-
A_84_P837010	ACA1	1.8161789	ACA1 (alpha carbonic anhydrase 1)
A_84_P857080	UBA1A	1.8082842	UBP1 interacting protein 1a (uba1a)

A_84_P11428	MYB20	3.2172334	myb20 (myb domain protein 20)
A_84_P537707	AT5G09880	2.7198632	RPP1 (recognition of peronospora parasitica 1)
A_84_P13008	AT2G04100	2.7174456	-
A_84_P17262	EDA16	2.535359	EDA16 (embryo sac development arrest 16)
A_84_P17411	AT1G20480	2.0291073	4-coumarate-coa ligase family protein
A_84_P785029	CBL10	1.7548419	CBL10 (calcineurin b-like 10; Calcium ion binding
A_84_P795384	VTE1	7.3837023	VTE1 (vitamin e deficient 1); tocopherol cyclase
A_84_P854905	ACR3	2.0657766	ACR3; amino acid binding
A_84_P15273	ATFER2	2.0654845	ATFER2 (ferritin 2)
A_84_P574087	AT1G28170	3.0536888	Sulfotransferase family protein
A_84_P11525	SVL2	3.047963	SVL2 (shv3-like 2); glycerophosphodiester
A_84_P805152	BT2	3.0204096	BT2 (btb and taz domain protein 2)
A_84_P855736	AT1G33770	3.0180187	Protein kinase family protein
A_84_P807362	AT2G14290	2.9881587	F-box family protein
A_84_P807337	AT4G02420	2.9837606	Lectin protein kinase, putative
A_84_P20343	ANTR2	2.9834993	ANTR2; inorganic phosphate transmembrane
A_84_P15599	AGL31	2.9681594	AGL31 (agamous like mads-box protein 31)
A_84_P855937	AtHSD5	2.946355	ATHSD5 (hydroxysteroid dehydrogenase 5)
A_84_P19531	ACS4	2.945571	ACS4 (1-aminocyclopropane-1-carboxylate synthase 4)
A_84_P806550	HMG1	1.7033826	HMG1 (hydroxy methylglutaryl coa reductase 1)
A_84_P806475	SDG37	1.7011533	SDG37; zinc ion binding
A_84_P806587	GC5	1.7011417	GC5 (golgin candidate 5); protein binding
A_84_P806496	UGP3	1.698138	UGP3 (udp-glucose pyrophosphorylase 3)
A_84_P806597	AT5G50130	1.6956713	Short-chain dehydrogenase/reductase (sdr) family protein
A_84_P21372	AT-HSFB3	2.9454627	AT-HSFB3; DNA binding / transcription factor
A_84_P19062	AT5G02950	2.9327846	PWWP domain-containing protein
A_84_P806907	AT5G56810	2.918799	F-box family protein
A_84_P14291	NAC105	2.9181292	NAC105 (nac domain containing protein 105)
A_84_P806923	ARL	2.8948483	ARL (argos-like)
A_84_P801305	AT5G24930	2.8700776	Zinc finger (b-box type) family protein
A_84_P18505	-	2.8663468	-
A_84_P801201	AT1G06360	2.8585851	Fatty acid desaturase family protein

A_84_P808787	PHT4;1	2.1907277	PHT4;1; carbohydrate transmembrane transporter
A_84_P807230	AT2G36320	1.7950294	Zinc finger (an1-like) family protein
A_84_P807241	JAZ5	1.792867	JAZ5 (jasmonate-zim-domain protein 5)
A_84_P14351	AT5G47730	1.7870816	SEC14 cytosolic factor
A_84_P297434	AT5G64270	1.651513	Splicing factor, putative
A_84_P813537	AT1G33470	3.4704084	RNA recognition motif (rrm)-containing protein
A_84_P856027	AT4G26370	3.2393646	Antitermination nusb domain-containing protein
A_84_P807322	AT1G04570	3.111556	Integral membrane transporter family protein
A_84_P18150	AT2G02620	2.737713	DC1 domain-containing protein / phd finger protein-related
A_84_P21687	AT1G07430	1.9510984	Protein phosphatase 2c, putative / pp2c, putative
A_84_P15540	AT2G47690	1.9509714	NADH-ubiquinone oxidoreductase-related
A_84_P808747	AT1G76820	1.9374304	GTP binding / gtpase
A_84_P19306	AT4G00250	1.9367101	DNA-binding storekeeper protein-related
A_84_P22988	CLC-F	1.5773581	CLC-F (chloride channel f); ion channel
A_84_P813598	IRE1A	1.8922155	IRE1a; endoribonuclease/ kinase
A_84_P808639	ATRABA2B	1.8942823	ATRABA2B (arabidopsis rab gtpase homolog a2b)
A_84_P813613	AT4G20770	1.934659	Pentatricopeptide (ppr) repeat-containing protein
A_84_P849716	IAA6	1.9339902	IAA6 (indole-3-acetic acid 6); transcription factor
A_84_P19190	AT3G27290	3.6022973	F-box family protein-related
A_84_P828541	NUA	1.8275455	NUA (nuclear pore anchor)
A_84_P112042	AT2G28720	2.273657	Histone h2b, putative
A_84_P792976	AT5G46250	1.6661881	RNA recognition motif (rrm)-containing protein
A_84_P769733	CYP79F2	7.963381	CYP79F2; oxidoreductase, acting on paired donors
A_84_P14056	AT1G72030	3.2250953	GCN5-related n-acetyltransferase (gnat) family protein
A_84_P847878	AT1G78310	3.219627	VQ motif-containing protein
A_84_P16211	AT1G19100	1.5166435	ATP-binding region
A_84_P55970	RCD1	3.4091544	RCD1 (radical-induced cell death1)
A_84_P810749	AT5G24930	2.5335207	Zinc finger (b-box type) family protein
A_84_P759880	ATGA2OX4	4.2057495	ATGA2OX4 (gibberellin 2-oxidase 4)
A_84_P16497	-	4.006761	-
A_84_P87569	AT3G01890	2.0183947	SWIB complex baf60b domain-containing protein
A_84_P297854	AT5G56700	1.8384281	F-box protein-related

A_84_P14757	AT1G71010	1.8376503	Phosphatidylinositol-4-phosphate 5-kinase family protein
A_84_P21906	RCA	1.732071	RCA (rubisco activase)
A_84_P11139	-	2.7952409	-
A_84_P160473	RPP5	2.7732508	RPP5 (recognition of peronospora parasitica 5)
A_84_P820984	AT1G72070	2.8099487	DNA heat shock n-terminal domain-containing protein
A_84_P822530	PSBP-1	3.7140174	PSBP-1 (photosystem ii subunit p-1)
A_84_P10836	AGL31	2.829855	AGL31 (agamous like mads-box protein 31)
A_84_P13548	DRT112	2.8244522	DRT112; copper ion binding / electron carrier
A_84_P816826	HSP91	2.8130636	HSP91; atp binding
A_84_P16417	AT3G17400	2.8076878	F-box family protein
A_84_P837376	DRT112	2.8008063	DRT112; copper ion binding / electron carrier
A_84_P22691	AT3G29380	2.7720966	Transcription factor iib (tfiib) family protein
A_84_P831332	-	2.6252358	-
A_84_P12510	AT1G55530	2.2959783	Zinc finger (c3hc4-type ring finger) family protein
A_84_P19359	ATTPS8	2.1821764	ATTPs8; alpha,alpha-trehalose-phosphate synthase
A_84_P22980	LUT2	2.1076748	LUT2 (lutein deficient 2); lycopene epsilon cyclase
A_84_P19649	AT5G34870	1.8165131	Zinc knuckle (cchc-type) family protein
A_84_P846461	-	1.7145913	-
A_84_P819590	AT1G80790	1.7145678	XH/XS domain-containing protein
A_84_P17919	AT3G49710	1.5126256	Pentatricopeptide (ppr) repeat-containing protein
A_84_P800779	AT5G37720	1.5015405	RNA and export factor-binding protein, putative
A_84_P122882	AT3G19330	1.932322	Polyadenylate-binding protein-related / pabp-related
A_84_P16491	LBD37	1.7123203	LBD37 (lob domain-containing protein 37)
A_84_P794016	RPP4	6.541517	RPP4 (recognition of peronospora parasitica 4); lrr domain binding
A_84_P784860	CR88	1.5068321	CR88; atp binding
A_84_P23769	PSAE-1	3.762242	PSAE-1 (psa e1 knockout); catalytic
A_84_P13627	AT5G64970	1.5954062	Mitochondrial substrate carrier family protein
A_84_P731627	AT2G38300	6.1477866	DNA binding / transcription factor
A_84_P764530	XIB	2.1434178	XIB (myosin xi b); motor
A_84_P857022	KAN3	2.1412554	KAN3 (kanadi 3); dna binding / transcription factor
A_84_P12443	AT1G66620	2.1322515	Seven in absentia (sina) protein, putative
A_84_P578925	ATCAD4	1.8843174	ATCAD4; cinnamyl-alcohol dehydrogenase

A_84_P560775	EMB3013	1.8777549	EMB3013 (embryo defective 3013)
A_84_P76294	-	1.8747813	-
A_84_P268330	AT4G33150	1.8742735	Lysine-ketoglutarate reductase/saccharopine
A_84_P121202	AT5G45190	1.8737229	ATCLC-a (chloride channel a)
A_84_P127351	ATCLC-A	1.8725911	NRAMP3 (natural resistance-associated macrophage protein)
A_84_P197914	NRAMP3	1.8725194	Carbohydrate transmembrane transporter
A_84_P607167	AT1G08900	1.8714632	Binding / ubiquitin-protein ligase
A_84_P834883	AT2G23140	1.8674778	SEC14 cytosolic factor, putative
A_84_P823769	AT5G47730	1.8647004	SEC14 cytosolic factor
A_84_P244005	-	1.8645735	-
A_84_P542592	AT3G21360	1.8599224	Electron carrier/ oxidoreductase
A_84_P533104	AT5G45160	1.8585256	Root hair defective 3 gtp-binding (rhd3) family protein
A_84_P22757	AT2G30060	1.8548793	RAN-binding protein 1b (ranbp1b)
A_84_P559441	HEMD	1.8541924	HEMD; uroporphyrinogen-iii synthase
A_84_P258200	-	1.5980586	-
A_84_P230699	RTFL2	5.479492	RTFL2 (rotundifolia like 2)
A_84_P246045	PSB28	5.426744	PSB28 (photosystem ii reaction center psb28 protein)
A_84_P19183	CYP71B32	5.317459	CYP71b32
A_84_P21759	PHT2;1	5.316656	PHT2;1 (phosphate transporter 2;1)
A_84_P16258	AT3G28330	5.2320056	F-box family protein-related
A_84_P12897	UGT84A2	5.2023306	UGT84A2; UDP-glycosyltransferase
A_84_P839899	AT4G34400	5.177777	DNA binding / transcription factor
A_84_P815436	AT5G64700	4.3959293	Nodulin MTN21 family protein
A_84_P16084	AT4G18330	3.2455559	Eukaryotic translation initiation factor 2 subunit 3, putative
A_84_P19561	NAC105	3.2127464	NAC105 (nac domain containing protein 105)
A_84_P10686	AT3G18160	3.2006216	Peroxin-3 family protein
A_84_P16876	AT5G59010	2.7589738	Protein kinase-related
A_84_P757006	AT5G38780	2.7459266	S-adenosyl-l-methionine:carboxyl methyltransferase
A_84_P19875	AT2G43610	2.7438862	Glycoside hydrolase family 19 protein
A_84_P285920	AT4G18375	2.0931988	KH domain-containing protein
A_84_P22938	AT3G06270	1.6806159	Protein phosphatase 2c, putative / pp2c, putative
A_84_P832854	CRD1	4.521229	CRD1 (copper response defect 1)

A_84_P21264	VPS46.2	1.6879648	VPS46.2
A_84_P810552	AT3G07720	1.6821615	Kelch repeat-containing protein
A_84_P24083	-	1.6112761	-
A_84_P807838	NRAMP6	3.6067839	NRAMP6; inorganic anion transmembrane
A_84_P15349	AT1G63880	3.5057073	Disease resistance protein (tir-nbs-lrr class)
A_84_P17021	AGL17	1.897796	AGL17 (agamous-like 17); transcription factor
A_84_P20420	ATMDAR1	1.7447078	Monodehydroascorbate reductase, putative
A_84_P785553	ATARCA	1.601581	ATARCA; nucleotide binding
A_84_P12217	ADF10	1.5274327	ADF10 (actin depolymerizing factor 10); actin binding
A_84_P17626	AT4G25835	1.5274187	AAA-type atpase family protein
A_84_P806238	EMB3011	1.5244484	EMB3011 (embryo defective 3011); helicase/ nucleic acid binding
A_84_P23860	AT1G49590	1.5241748	Formin-binding protein-related
A_84_P826232	RHA3A	1.5201204	RHA3a; protein binding / zinc ion binding
A_84_P20834	AT5G50370	1.5173149	Adenylate kinase, putative
A_84_P12586	PSAG	3.6101985	PSAG (photosystem i subunit g)
A_84_P16902	AT5G10350	1.571948	Polyadenylate-binding protein family protein / pabp family protein
A_84_P10813	AT5G47430	1.9297729	Zinc ion binding
A_84_P297614	AT2G46620	3.2395976	AAA-type atpase family protein
A_84_P17835	RPL18AA	1.5537076	RPL18aa (60s ribosomal protein l18a-1)
A_84_P819664	AT1G62390	1.5533825	RPL18AA (60s ribosomal protein l18a-1)
A_84_P847632	PSAH2	8.68869	-
A_84_P785928	-	6.290778	-
A_84_P257240	AT5G37760	4.446328	DNAj heat shock n-terminal domain-containing protein
A_84_P63724	PIF4	4.3649282	PIF4 (phytochrome interacting factor 4)
A_84_P267120	PDF1.5	4.0194936	PDF1.5 (plant defensin 1.5)
A_84_P579017	AT5G51610	3.4424207	Ribosomal protein l11 family protein
A_84_P557066	WOX5	3.4311886	WOX5 (wuschel related homeobox 5); transcription factor
A_84_P244835	SPA3	2.655509	SPA3 (spa1-related 3); protein binding
A_84_P813342	AT1G20480	2.4606316	4-coumarate--coa ligase family
A_84_P73044	AT5G17230	2.28149	Phytoene synthase (psy)
A_84_P224379	-	2.242186	-
A_84_P831629	RGE1	2.2054427	RGE1 (retarded growth of embryo 1)

A_84_P17584	VTC2	2.1182199	VTC2 (vitamin c defective 2
A_84_P51520	MIZ1	2.076676	MIZ1 (mizu-kussei 1)
A_84_P789206	VAL3	1.9165453	VAL3 (vp1/abi3-like 3); transcription factor
A_84_P835822	AT2G36320	1.8132439	Zinc finger (an1-like) family protein
A_84_P18890	AT1G19970	1.7747484	ER lumen protein retaining receptor family protein
A_84_P176434	AT2G46610	1.7722604	Arginine/serine-rich splicing factor, putative
A_84_P590597	ATEP3	1.7703978	ATEP3; chitinase
A_84_P856502	G6PD5	1.7320751	G6PD5 (glucose-6-phosphate dehydrogenase 5)
A_84_P765122	CRR2	1.6791784	CRR2 (chlororespiratory reduction 2)
A_84_P576786	AT5G57210	1.6788499	Microtubule-associated protein-related
A_84_P265650	AT2G03270	1.677211	DNA-binding protein, putative
A_84_P606193	AT2G37160	1.6429169	Transducin family protein / wd-40 repeat family protein
A_84_P543942	AT1G47770	1.624696	Beta-galactosidase
A_84_P752369	-	1.5980551	-
A_84_P230289	GAPC2	1.5950452	GAPC2 (glyceraldehyde-3-phosphate dehydrogenase c2)
A_84_P610807	ETR2	1.5918363	ETR2 (ethylene response 2)
A_84_P547587	GLX2-1	1.5053986	GLX2-1 (glyoxalase 2-1); hydroxyacylglutathione hydrolase
A_84_P586094	AT4G24810	2.598206	ABC1 family protein
A_84_P538884	AT5G50915	2.5832036	Basic helix-loop-helix (bhlh) family protein
A_84_P766880	AT3G28345	2.5827277	ABC transporter family protein
A_84_P17604	RTV1	2.5817091	RTV1 (related to vernalization1 1); dna binding
A_84_P575019	AT2G36460	2.579369	Fructose-bisphosphate aldolase, putative
A_84_P213138	AT5G29000	2.5665293	MYB family transcription factor
A_84_P567661	AT2G43610	2.5641394	Glycoside hydrolase family 19 protein
A_84_P212598	MYB4	2.5615077	MYB4; dna binding / transcription factor
A_84_P752474	AT2G47520	2.5596569	AP2 domain-containing transcription factor, putative
A_84_P21932	PSBR	2.4926212	PSBR (photosystem ii subunit r)
A_84_P84229	AT3G45440	1.8225067	Lectin protein kinase family protein
A_84_P556476	UBC35	1.7839037	UBC35 (ubiquitin-conjugating enzyme 35)
A_84_P525328	ADOF1	1.734767	ADOF1; dna binding / transcription factor
A_84_P587556	CPuORF19	1.679894	CPUORF19 (conserved peptide upstream open reading frame 19)
A_84_P15698	AT1G56120	2.1561825	Kinase

A_84_P204928	-	2.086136	-
A_84_P812689	ZIP11	2.080834	ZIP11 (zinc transporter 11 precursor
A_84_P812703	ZIP11	2.0806613	ZIP11 (zinc transporter 11 precursor)
A_84_P806316	AT1G23205	6.1976776	Invertase/pectin methylesterase inhibitor family protein
A_84_P216738	AT2G33550	2.0080192	GT-2-related
A_84_P14803	ATNUDX15	1.9829987	ATNUDX15 (nudix hydrolase homolog 15); hydrolase
A_84_P856161	AT1G74320	1.8861817	Choline kinase, putative
A_84_P16044	AT5G05880	1.5512738	UDP-glucoronosyl/udp-glucosyl transferase family protein
A_84_P840671	-	2.634459	-
A_84_P786139	AT5G35100	1.6906191	Peptidyl-prolyl cis-trans isomerase
A_84_P14423	AT1G77470	1.542686	Replication factor c 36 kda, putative
A_84_P14108	AT3G55480	1.6080872	Adaptin family protein
A_84_P823401	AT5G47030	1.5293968	ATP synthase delta' chain, mitochondrial
A_84_P839062	ROPGEF4	10.932403	ROPGEF4 (rho guanyl-nucleotide exchange factor 4)
A_84_P795186	AT4G00390	6.908919	Transcription regulator
A_84_P154615	AtbZIP75	5.7807527	ATBZIP75 (basic leucine-zipper 75); dna binding / transcription
A_84_P766709	SPL4	5.7454987	SPL4 (squamosa promoter binding protein-like 4)
A_84_P834919	AT1G70460	5.598516	Protein kinase, putative
A_84_P75104	AtGDU6	4.69383	ATGDU6 (glutamine dumper 6)
A_84_P268940	AT3G61490	4.6721697	Glycoside hydrolase family 28 protein
A_84_P11730	BT2	4.475031	BT2 (btb and taz domain protein 2)
A_84_P154775	RBCS1A	4.416333	RBCS1a (ribulose bisphosphate carboxylase small chain)
A_84_P561083	HDG11	4.2624683	HDG11 (homeodomain glabrous 11)
A_84_P10029	CPUORF47	4.188322	CPUORF47 (open reading frame)
A_84_P15953	HPR	3.9686732	HPR; glycerate dehydrogenase/ poly(u) binding
A_84_P796358	AT5G43300	3.9017804	Glycerophosphodiester phosphodiesterasepsbp-1
A_84_P17258	PSBP-1	3.6553955	Photosystem ii subunit p-1); poly(u) binding
A_84_P767978	-	3.6258483	-
A_84_P296274	CKX4	3.3158383	CKX4 (cytokinin oxidase 4); amine oxidase
A_84_P858600	AT4G01050	3.254794	Hydroxyproline-rich glycoprotein family protein
A_84_P15660	DRT112	3.1751401	DRT112; copper ion binding / electron carrier
A_84_P851998	GPAT6	3.1210694	GPAT6 (glycerol-3-phosphate acyltransferase 6)

A_84_P834538	-	3.0702665	-
A_84_P182114	AT2G43100	3.065988	Aconitase containing protein
A_84_P182664	AT1G61660	2.7112756	Basic helix-loop-helix (bhlh) family protein
A_84_P226299	ATBAG5	2.7100396	ATBAG5 (bcl-2-associated athanogene 5)
A_84_P10452	AT5G15910	2.619408	Dehydrogenase-related
A_84_P213918	RING1B	2.6095343	Ring1b (ring 1b)
A_84_P20958	UGT78D2	2.5161498	UGT78D2 (udp-glucosyl transferase 78d2)
A_84_P93349	CYCA1;2	2.468953	CYCA1;2 (cyclin a1;2)
A_84_P839051	AT1G76010	2.4528465	Nucleic acid binding
A_84_P23880	AT5G47050	2.4096851	Protein binding / zinc ion binding
A_84_P769649	AT1G76590	2.3898773	Zinc-binding family protein
A_84_P842818	MAF3	2.386465	MAF3 (mads affecting flowering 3)
A_84_P812152	AT1G80440	2.2410126	Kelch repeat-containing f-box family protein
A_84_P70844	MLO7	2.2381427	MLO7 (mildew resistance locus o 7)
A_84_P763856	ASK7	2.235628	ASK7 (arabidopsis skp1-like 7)
A_84_P13967	AT5G46410	2.1889992	NLI interacting factor (nif) family protein
A_84_P518026	AT5G27650	2.1744568	PWWP domain-containing protein
A_84_P212288	UGT75B1	2.144092	UGT75B1 (udp-glucosyltransferase 75b1)
A_84_P784970	AT3G01210	2.0772414	Nucleic acid binding / oxidoreductase
A_84_P852628	AT4G02180	2.0433893	DC1 domain-containing protein
A_84_P786940	EOL2	2.0110612	EOL2 (eto1-like 2); binding / protein binding
A_84_P156845	AZF2	2.0100927	AZF2 (arabidopsis zinc-finger protein)
A_84_P283770	AT4G19510	1.9950163	Disease resistance protein
A_84_P831478	-	1.9587361	-
A_84_P290374	-	1.7817882	-
A_84_P88039	RAP2.4	1.7517141	DGL (dongle); triacylglycerol lipase
A_84_P21340	SCPL11	1.7493818	GAPB (glyceraldehyde-3-phosphate dehydrogenase)
A_84_P12972	HSFA7A	1.7371283	-
A_84_P147788	AML2	1.6892545	GT-2-related
A_84_P14176	AGP22	1.6499454	GA20ox1; gibberellin 20-oxidase
A_84_P865032	AT3G27540	1.641478	LBD25 (lob domain-containing protein 25)
A_84_P17624	WRKY69	1.640235	Protein binding / zinc ion binding

A_84_P842009	AT5G61590	1.640146	-
A_84_P793535	AT2G21500	1.6087049	OBP3 (obf-binding protein 3); dna binding / transcription factor
A_84_P231489	AT1G53780	1.6083769	Zinc finger (b-box type) family protein
A_84_P199564	AT4G34050	1.5783312	Triacylglycerol lipase
A_84_P22126	AT3G09760	1.572802	UGT71b5 (udp-glucosyl transferase 71b5)
A_84_P15145	AT1G63080	1.54298	SIP4 (sos3-interacting protein 4); kinase/ protein kinase
A_84_P795523	ATPUP14	1.5047837	RPP8 (recognition of peronospora parasitica 8)
A_84_P831561	BLH2	1.9750942	Hydroxyproline-rich glycoprotein family protein
A_84_P838187	ZIP6	1.9706864	-
A_84_P93039	AT4G16960	2.3645213	-
A_84_P770344	EMS1	7.871726	EMS1 (excess microsporocytes1
A_84_P805825	AT3G58520	1.6458458	Ubiquitin thiolesterase
A_84_P14039	AT2G48100	1.6452198	Exonuclease family protein
A_84_P22130	-	3.8777332	-
A_84_P19938	AtATG18h	1.7099037	ATATG18h
A_84_P20747	AT1G21100	4.4712873	O-methyltransferase, putative
A_84_P19012	-	4.4586015	-
A_84_P12962	DGL	4.4554825	DGL (dongle); triacylglycerol lipase
A_84_P751290	AT1G12130	4.4448977	Flavin-containing monooxygenase family protein
A_84_P17719	GAPB	4.4334207	GAPB (glyceraldehyde-3-phosphate dehydrogenase b)
A_84_P23464	-	4.198185	-
A_84_P602765	AT2G33550	2.0017102	GT-2-related
A_84_P81579	GA20OX1	1.8873277	GA20ox1; gibberellin 20-oxidase
A_84_P19726	LBD25	3.6853487	LBD25 (lob domain-containing protein 25)
A_84_P14739	AT1G60610	1.626085	Protein binding / zinc ion binding
A_84_P799867	-	7.253167	-
A_84_P16935	OBP3	2.2107768	OBP3 (obf-binding protein 3)
A_84_P18663	AT5G24930	2.6055794	Zinc finger (b-box type) family protein
A_84_P22284	AT5G42930	1.8843508	Triacylglycerol lipase
A_84_P304780	UGT71B5	1.6467218	UGT71b5 (udp-glucosyl transferase 71b5)
A_84_P799565	SIP4	6.515901	SIP4 (sos3-interacting protein 4)
A_84_P847580	RPP8	2.5044444	RPP8 (recognition of peronospora parasitica 8)

A_84_P270170	AT4G01050	2.4998446	Hydroxyproline-rich glycoprotein family protein
A_84_P793182	-	6.171985	-
A_84_P15108	-	2.104002	-
A_84_P23356	AT1G32580	1.6260028	Plastid developmental protein dag, putative
A_84_P11965	CYP96A12	1.9946748	CYP96A12; electron carrier/ heme binding
A_84_P310273	ISA3	1.8079214	ISA3 (isoamylase 3); alpha-amylase
A_84_P19129	-	1.9675645	-
A_84_P786064	AT5G05550	1.9628015	Transcription factor
A_84_P826840	AT1G73180	1.6348361	Eukaryotic translation initiation factor-related
A_84_P13900	AT5G50370	1.7421364	Adenylate kinase, putative
A_84_P18043	AT5G55530	1.7408211	C2 domain-containing protein
A_84_P845705	AT5G19390	1.7407238	Adenylylsulfate kinase, putative
A_84_P272220	AT3G24760	1.7404264	Glycosyl transferase-related
A_84_P23682	P5CS2	1.7143025	SAM-2 (s-adenosylmethionine synthetase 2)
A_84_P23359	AT4G29030	4.247763	ATBZIP52 (basic leucine zipper 52); dna binding
A_84_P21800	ATCHX19	1.6663969	Cupin family protein
A_84_P23846	AT2G29530	1.625192	Phenazine biosynthesis phzc/phzf family protein
A_84_P851011	ATMRP4	1.705405	Zinc finger (c3hc4-type ring finger) family protein
A_84_P825227	AT1G80570	1.621148	ATATH13; transporter
A_84_P155475	AT3G03900	2.0437484	Pentatricopeptide (ppr) repeat-containing protein
A_84_P11781	AT4G19900	1.6231929	DNA repair atpase-related
A_84_P21357	SAM-2	2.1122673	Organic anion transmembrane transporter
A_84_P853131	AtbZIP52	1.6691712	MYB family transcription factor
A_84_P22036	AT4G36700	1.7539674	COR47 (cold-regulated 47)
A_84_P10539	AT1G03210	1.7141116	C2 domain-containing protein
A_84_P818785	AT2G39100	2.0047293	Diacylglycerol kinase, putative
A_84_P11823	ATATH13	2.210791	Sec61 beta; protein transporter
A_84_P853697	AT3G04760	7.542692	Disease resistance protein (tir-nbs-lrr class), putative
A_84_P752180	AT2G24420	3.879769	Basic helix-loop-helix (bhlh) family protein (bhlh096)
A_84_P577102	AT5G11230	1.9817703	BHLH family protein
A_84_P859915	AT5G29000	2.657749	Translation initiation factor
A_84_P303930	COR47	1.753742	ATNUDT10 (nudix hydrolase homolog 10)

A_84_P17147	AT5G55530	1.5903281	CSP41A (chloroplast stem-loop binding, putative
A_84_P17026	AT2G18730	1.5515712	Disease resistance protein
A_84_P754376	SEC61_BETA	1.542641	SEC61 BETA, protein transporter
A_84_P12289	AT2G14080	1.8509637	Disease resistance protein, putative
A_84_P20362	AT1G72210	2.6324117	Basic helic loop-helix (bHLH) family protein
A_84_P157055	AT3G07920	1.5995451	Translation initiation factor
A_84_P14144	ATNUDT10	3.7988822	Oxidoreductase n-terminal domain-containing protein
A_84_P22521	CSP41A	3.4617345	Lectin protein kinase family protein
A_84_P17546	AT1G59218	1.850618	Heat shock protein binding
A_84_P13078	AT5G46070	1.5856375	Phosphoinositide binding
A_84_P855161	ProT3	2.5087764	PROT3 (proline transporter 3)
A_84_P16678	XIK	1.931789	XIK; motor/ protein binding
A_84_P15062	ATM2	1.6238513	ATM2 (myosin 2); motor
A_84_P17344	AGL16	1.5507238	AGL16 (agamous-like 16), transcription factor
A_84_P834169	AT1G66130	2.5214312	ALATS (ALANYL-tRNA synthase)
A_84_P840311	AT1G34300	1.8363743	-
A_84_P21661	AT2G41000	1.587019	F-box family protein (fbx3)
A_84_P204058	AT1G48090	1.8351334	GBF2 (g-box binding factor 2); dna binding
A_84_P20482	SURF1	1.7179066	HPR; glycerate dehydrogenase/ poly(u) binding
A_84_P818633	AT5G13770	4.4580584	-
A_84_P18005	PBP1	1.6670557	LBD7 (lob domain-containing protein 7)
A_84_P789465	ALATS	1.5349419	MYB family transcription factor
A_84_P807484	-	1.9545089	-
A_84_P15412	AT1G76920	1.892851	F-box family protein (fbx3)
A_84_P19924	GBF2	1.578294	GBF2 (g-box binding factor 2)
A_84_P764623	HPR	5.6150804	HPR (glycerate dehydrogenase)
A_84_P816461	-	3.6018176	-
A_84_P111252	LBD7	3.5439644	LBD7 (lob domain-containing protein 7)
A_84_P83659	AT5G58900	1.8462187	MYB family transcription factor
A_84_P12614	AGL17	1.7808697	AGL17 (agamous-like 17); transcription factor
A_84_P792498	SFGH	1.5048753	SFGH (s-formylglutathione hydrolase)
A_84_P763156	THI1	8.594463	THI1; protein homodimerization

A_84_P861121	AT5G17670	2.0589626	Hydrolase, acting on ester bonds
A_84_P18051	-	4.174794	-
A_84_P17138	MAF3	3.1212165	MAF3 (mads affecting flowering 3)
A_84_P763777	AT1G17210	1.9108201	Zinc ion binding
A_84_P10917	CYP722A1	1.6768019	CYP722a1
A_84_P836619	UPL2	2.2985072	UPL2 (ubiquitin-protein ligase 2)
A_84_P13660	LCR10	4.1819882	LCR10 (low-molecular-weight cysteine-rich 10)
A_84_P750656	FTSH1	3.7780383	FTSH1 (ftsh protease 1)
A_84_P549867	PSAG	3.6422417	PSAG (photosystem i subunit g)
A_84_P598794	PSBO2	3.6324449	PSBO2 (photosystem ii subunit o-2)
A_84_P17788	PSAG	3.630042	PSAG (photosystem i subunit g)
A_84_P838228	ACA1	3.3602154	ACA1 (alpha carbonic anhydrase 1)
A_84_P10489	GAPA	3.3595414	GAPA (glyceraldehyde 3-phosphate dehydrogenase a)
A_84_P18121	AT1G21100	2.7068527	O-methyltransferase, putative
A_84_P18836	AT3G05155	2.3969498	Sugar transporter, putative
A_84_P790868	ADOF1	2.3586545	ADOF1; DNA binding
A_84_P10333	GLT1	2.3581853	GLT1; glutamate synthase (nadh)
A_84_P737484	AT4G29590	1.9282318	Methyltransferase
A_84_P169943	AT4G27700	1.8110983	Rhodanese-like domain-containing protein
A_84_P90969	ATBPM6	1.718921	Atbpm6 (btb-poz and math domain 6)
A_84_P10470	-	1.6590723	-
A_84_P133525	ARAB-1	1.6579636	ARAB-1; carboxylesterase
A_84_P818939	AT3G62890	1.5834925	Binding
A_84_P22438	AXR1	1.5804037	AXR1 (auxin resistant 1)
A_84_P14786	AT-P4H-1	1.578889	AT-P4H-1 (a. thaliana p4h isoform 1)
A_84_P826247	AT1G28280	1.5629637	VQ motif-containing protein
A_84_P819117	AT1G14220	3.9529579	Ribonuclease t2 family protein
A_84_P23525	PSBP-1	3.911333	PSBP-1 (photosystem ii subunit p-1)
A_84_P10142	-	4.5865965	-
A_84_P509017	AT3G61490	3.9015436	Glycoside hydrolase family 28 protein
A_84_P10895	AT5G13510	1.5530773	Ribosomal protein l10 family protein
A_84_P834503	-	4.952603	-

A_84_P835481	AT1G66310	4.9354515	F-box family protein
A_84_P857938	ATEXO70H6	4.90259	ATEXO70H6 (exocyst subunit exo70 family protein h6)
A_84_P13132	GAPA-2	4.8488016	GAPA-2 (glyceraldehyde 3-phosphate dehydrogenase a)
A_84_P12639	SHM1	3.8957632	SHM1 (serine transhydroxymethyltransferase 1)
A_84_P828803	AT5G05530	3.8854885	Zinc finger (c3hc4-type ring finger) family protein
A_84_P19534	EMB1586	2.7275157	EMB1586 (embryo defective 1586)
A_84_P855014	RHA3B	2.4773297	RHA3B; protein binding / zinc ion binding
A_84_P10294	AT3G01890	2.2607467	SWIB complex baf60b domain-containing protein
A_84_P18234	AT5G47030	1.6569499	ATP synthase delta' chain, mitochondrial
A_84_P17848	SAM1	1.6346325	SAM1 (s-adenosylmethionine synthetase 1)
A_84_P75674	AT3G13470	1.5830625	Chaperonin, putative
A_84_P10278	SKP1	1.5560336	SKP1 (s phase kinase-associated protein 1)
A_84_P77139	PSAL	4.179284	PSAL (photosystem i subunit l)
A_84_P830406	AT3BETAHSD/D1	1.7106105	AT3BETAHSD/D1(betahydroxysteroid dehydrogenase)
A_84_P17828	AT2G17525	2.6033933	Pentatricopeptide (ppr) repeat-containing protein
A_84_P253455	ATRAB28	2.5990112	ATRAB28
A_84_P15999	AT3G53840	2.2765744	Protein kinase family protein
A_84_P851557	AT5G45360	1.7403299	F-box family protein
A_84_P255810	LHCA3	1.7389212	LHCA3; chlorophyll binding
A_84_P16085	WNK10	1.6526517	WNK10 (with no lysine kinase 10)
A_84_P867277	AT3G56080	4.445317	Dehydration-responsive protein-related
A_84_P10252	PSAD-1	2.5455916	PSAD-1 (photosystem i subunit d-1)
A_84_P763163	DRT112	3.1938114	DRT112; copper ion binding
A_84_P805176	AT3G60920	9.70107	Beige/beach domain-containing protein
A_84_P868105	AT4G35060	1.9878817	Heavy-metal-associated domain-containing protein
A_84_P21320	EMB1865	1.9872963	EMB1865 (embryo defective 1865)
A_84_P302000	TOM2B	1.5716519	TOM2B (tobamovirus multiplication 2b)
A_84_P848740	AT2G19910	3.6772628	RNA-dependent rna polymerase family protein
A_84_P13446	J8	1.9780428	J8; heat shock protein binding
A_84_P20245	SC3	1.7486124	SC3 (secretory carrier 3)
A_84_P760385	AT5G02600	2.534754	Heavy-metal-associated domain-containing protein
A_84_P788550	ERD15	1.5102168	ERD15 (early responsive to dehydration 15); protein binding

A_84_P812251	-	1.7359235	-
A_84_P807693	AT1G09510	3.4671884	Cinnamyl-alcohol dehydrogenase family / cad family
A_84_P13370	ATPSK4	3.4646792	ATPSK4 (phytosulfokine 4 precursor); growth factor
A_84_P23070	AT4G32630	2.0652373	ARF GTPASE activator/ zinc ion binding
A_84_P814018	AT1G51300	2.2188263	Acyl-protein thioesterase-related
A_84_P12931	-	2.2176929	-
A_84_P814026	-	2.2155037	-

Table A2 180 genes differentially expressed between the GR24-treated and control calli of the strigolactone biosynthesis mutant, *max4-1*

Array ID	Gene symbol	Fold change	Gene name
A_84_P806658	ATMS1	1.8572785	Methyltetrahydropteroyltriglutamate-Homocysteine
A_84_P23070	HMT3	2.8083653	HMT3; homocysteine
A_84_P153128	AT4G16980	4.199085	Arabinogalactan-protein family
A_84_P12256	AT1G56710	1.9505287	Glycoside hydrolase
A_84_P718448	AT5G35100	2.13486	Peptidyl-prolyl isomerase
A_84_P75674	AT4G03440	1.6499201	Ankyrin repeat family
A_84_P21073	AtPP2-B5	1.9764547	ATPP2-B5 (Phloem protein 2-B5)
A_84_P118722	SEC8	1.5693867	SEC8 (SUBUNIT OF EXO COM 8)
A_84_P762426	AT3G49668	1.5786675	SEC8 (SUBUNIT OF EXO COM8)
A_84_P822060	AT2G19430	1.6028212	Transducin family protein
A_84_P186444	TET10	1.7972996	TET10 (TETRASPANIN10)
A_84_P838356	AT5G53100	1.8093624	Oxidoreductase, putative
A_84_P10073	AT4G28040	2.2183247	Nodulin MtN21 family protein
A_84_P598794	AT1G66610	2.3208404	Seven in absentia (SINA) protein
A_84_P12617	ANAC038	2.3265238	ANAC038 (ARABIDOPSIS NAC)
A_84_P19176	AGO5	2.797974	AGO5 (ARGONAUTE 5)
A_84_P852904	SCR	4.8620477	SCR (SCARECROW)
A_84_P750656	SPL4	5.2124767	SPL4 (SQUAMOSA PROMOTER BINDING PROTEIN-
A_84_P14793	AT1G21890	7.0613446	LIKE 4)
A_84_P821477	RAD23	1.6378657	Nodulin MtN21 family protein
A_84_P21962	AT2G31830	1.6027796	RAD23; damaged DNA binding
A_84_P750021	-	3.0960138	Endonuclease/exonuclease/family
A_84_P799083	-	1.5340422	-
A_84_P23051	AT3G04050	1.8229796	-
A_84_P20399	DFL2	1.5331136	Pyruvate kinase, putative
A_84_P13287	AT1G75580	2.5817692	DFL2 (DWARF IN LIGHT 2)
A_84_P12742	AT3G45770	1.5537076	Auxin-responsive protein, putative
A_84_P12289	AT1G06360	2.3438716	Oxidoreductase, zinc-binding family

A_84_P853697	-	1.6867663	Fatty acid desaturase family protein
A_84_P10221	ATGSL09	1.9073884	-
A_84_P17864	CYP86A1	5.9231915	ATGSL09 (glucan synthase-like 9);
A_84_P15266	AT1G74460	2.6302423	1,3-beta-glucan synthase
A_84_P182054	AT5G37690	3.4899933	Cytochrome P450
A_84_P145709	AT1G13130	2.366971	GDSL-motif lipase/hydrolase
A_84_P14218	KOR2	3.2896936	GDSL-motif lipase/hydrolase
A_84_P15108	AT1G23205	1.5264796	Glycosyl hydrolase family
A_84_P11258	AT5G61290	6.261394	KOR2; catalytic/ hydrolase
A_84_P16935	AT1G55260	6.231214	Invertase/pectin inhibitor
A_84_P22130	SURF1	2.1149962	Flavin-containing monooxygenase
A_84_P93039	AT2G47690	1.6026155	SURF1 (SURFEIT 1)
A_84_P757817	AT2G42388	1.5781612	NADH-ubiquinone oxidoreductase
A_84_P822067	AT3G10915	1.5983373	MiscRNA
A_84_P100516	AT4G22990	1.6083524	Reticulon family protein
A_84_P10029	AT4G13840	1.669094	SPX (SYG1/Pho81/XPR1)
A_84_P538464	-	1.7277119	Transferase family protein
A_84_P102076	AT5G64690	1.7478136	-
A_84_P10157	AT5G06330	1.8826755	Neurofilament triplet
A_84_P290374	AT4G00250	2.313464	Hairpin-responsive protein
A_84_P289784	AT1G61840	2.3177717	DNA-binding storekeeper
A_84_P12972	AT4G36700	2.987304	DC1 domain-containing
A_84_P855542	AT5G38100	3.0794513	Cupin family protein
A_84_P784935	AT1G15330	3.1213093	Methyltransferase-related
A_84_P19777	AT5G63560	3.2703028	CBS domain-containing protein
A_84_P518026	LCR10	3.5465212	transferase family protein
A_84_P549559	AT5G38100	3.682312	LCR10 (cysteine-rich 10)
A_84_P11660	AT2G07760	4.059809	Methyltransferase-related
A_84_P760097	AtGRF4	4.1287146	Zinc knuckle family protein
A_84_P759212	AT3G45840	4.2409325	ATGRF4 (GROWTH-REG FACTOR)
A_84_P812160	-	4.8627143	Protein binding / zinc ion binding
A_84_P835186	AT3G06778	5.673519	-

A_84_P14803	AT4G29020	3.4564016	Heat shock protein binding
A_84_P810056	AT5G46730	4.729993	Glycine-rich protein
A_84_P567661	AT2G17525	1.5206523	Glycine-rich protein
A_84_P611459	AT3G11460	1.7881678	Pentatricopeptide (PPR)
A_84_P20653	AT5G45220	1.5263504	Pentatricopeptide (PPR)
A_84_P754199	AT1G67328	1.5085037	Toll-Interleukin-Resistance (TIR)
A_84_P11766	AT3G13440	1.5395254	MiscRNA
A_84_P516219	AT1G76170	1.5682021	Methyltransferase/ nucleic acid binding
A_84_P835822	emb2739	1.6016382	ATP binding
A_84_P785928	-	1.6217169	EMB2739 (embryo defective 2739)
A_84_P789206	-	1.8520775	-
A_84_P844741	-	2.015918	-
A_84_P770350	-	2.077228	-
A_84_P547587	-	2.2238176	-
A_84_P205048	AT5G01080	3.759021	-
A_84_P557066	RTFL2	4.5265803	Beta-galactosidase
A_84_P750901	AT1G59171	5.9438424	RTFL2 (ROTUNDIFOLIA LIKE 2)
A_84_P752369	-	6.4526043	ATP binding
A_84_P766445	OVA2	1.6923866	-
A_84_P806238	AT2G46620	3.8958752	OVA2 (ovule abortion 2)
A_84_P13514	AT2G28040	1.5275271	AAA-type ATPase family protein
A_84_P868560	AT3G54940	1.9562862	Aspartyl protease family protein
A_84_P19126	-	1.7593483	Cysteine-type endopeptidase
A_84_P556286	SCPL15	8.36747	-
A_84_P818269	XSP1	1.5020202	SCPL15 (serine carboxypeptidase-like)
A_84_P285920	AT3G48030	1.5646942	XSP1 (xylem serine peptidase 1)
A_84_P839899	AT1G04790	1.5647645	Hypoxia-responsive family
A_84_P282890	AT5G47610	2.9223702	Zinc finger (C3HC4-type RING finger)
A_84_P558547	AT5G65850	1.742817	Zinc finger (C3HC4-type RING finger)
A_84_P542592	AT3G17400	2.4348881	F-box family protein
A_84_P589759	AT1G20795	3.6251564	WNK10 (WITH NO LYSINE KINASE)
A_84_P800779	WNK10	1.5496813	Protein kinase family

A_84_P160473	AT1G65250	3.0370164	ATP binding / kinase
A_84_P22512	AT5G35580	1.8857012	Ribosomal protein L11 family protein
A_84_P14056	AT5G51610	2.9111872	RPL12-A (RIBOSOMAL PROTEIN L)
A_84_P801048	RPL12-A	2.390038	TIM9; transmembrane
A_84_P814234	TIM9	1.5873966	GAPB (GLYCERALDEHYDE-P-DEHY)
A_84_P811397	GAPB	7.2996554	PSAD-1 (photosystem I subunit D-1)
A_84_P805815	PSAD-1	1.8806952	CAB1 (CHLOROPHYLL A/B BINDING)
A_84_P806496	CAB1	7.092637	-
A_84_P769873	-	1.6113896	Ribonuclease T2 family protein
A_84_P11428	AT1G14220	3.3522928	LBD7 (LOB DOMAIN-CONTAINING)
A_84_P506433	LBD7	3.878005	DNA binding / transcription factor
A_84_P10100	AT4G34400	1.6937702	DNA binding / transcription factor
A_84_P238643	AT4G31615	2.1628933	DNA binding / transcription factor
A_84_P830495	AT4G34400	2.3372152	ATBZIP52 (basic leucine zipper 52)
A_84_P20336	AtbZIP52	1.6212552	EEL (ENHANCED EM LEVEL)
A_84_P13542	EEL	1.9016128	ATBZIP48 (leucine-zipper 48)
A_84_P16999	AtbZIP48	2.2389193	Nucleic acid binding / zinc ion binding
A_84_P859366	AT5G18550	1.6491096	Zinc finger (C2H2 type) family protein
A_84_P10335	AT5G66730	1.6788055	Zinc finger (C2H2 type) family protein
A_84_P786186	AT1G14580	2.3383036	ANL2 (ANTHOCYANINLESS 2)
A_84_P12824	ANL2	2.087379	HDG12 (HOMEODOMAIN GLAB)
A_84_P15294	HDG12	2.9989905	MBD9; DNA binding / methyl-CpG bind
A_84_P837023	MBD9	2.0694897	Kelch repeat-containing protein
A_84_P138519	AT3G27220	1.7148795	MYB22 (myb domain protein 22)
A_84_P18744	MYB22	3.3020654	PHD finger family protein
A_84_P834703	AT5G22760	1.5907376	Transcriptional regulator-related
A_84_P836098	AT1G02080	1.9182914	Glycine-rich protein
A_84_P808742	AT4G29030	3.6023014	Zinc finger (CCCH-type) family
A_84_P845392	AT1G75340	1.5560508	CSP41A (CHLORO STEM-LOOP)
A_84_P815131	CSP41A	2.299633	WRKY38; transcription factor
A_84_P11143	WRKY38	2.647699	WRKY38; transcription factor
A_84_P845967	WRKY38	2.8332934	ATTPS-CIN (terpene synthase-like)

A_84_P156185	ATTPS-CIN	2.2218132	ATTPS-CIN (terpene synthase-like)
A_84_P784586	ATTPS-CIN	4.3604474	Transferase family protein
A_84_P15762	AT4G31910	6.334733	ATCAD4; cinnamyl-alcohol dehy
A_84_P18353	ATCAD4	1.7106467	CAD6 (CINNAMYL ALCOHOL DEHY)
A_84_P15784	CAD6	4.189031	Caffeoyl-CoA 3-O-methyltransferase
A_84_P17662	AT4G34050	1.644637	-
A_84_P709830	-	1.7871302	CBL10 (CALCINEURIN B-LIKE 10)
A_84_P843889	CBL10	1.9812053	GTP-binding protein (SAR1A)
A_84_P22197	AT1G02620	1.5495958	RNA recognition motif (RRM)
A_84_P824926	AT4G12640	1.8812181	Lectin protein kinase family
A_84_P10240	AT5G42120	1.6361089	Lectin protein kinase, putative
A_84_P266040	AT4G02420	4.7446513	Leucine-rich repeat transmembrane
A_84_P10860	AT3G47580	1.8214965	ATP binding / kinase
A_84_P21908	AT1G51830	5.567448	CLV3 (CLAVATA3); kinase activator
A_84_P132365	CLV3	7.3434377	Protein kinase family protein
A_84_P836875	AT3G13690	1.7521802	Protein kinase, putative
A_84_P16183	AT1G70460	5.816233	AOC1 (ALLENE OXIDE CYCLASE 1)
A_84_P786499	AOC1	1.6422489	ERD2 (EARLY-RESP TO DEHY)
A_84_P17172	ERD2	1.6740168	Dehydrin, putative
A_84_P12005	AT4G38410	1.690157	Pollen Ole e 1 allergen and extensin
A_84_P116942	AT4G02270	1.8077143	GLP6 (GERMIN-LIKE PROTEIN 6)
A_84_P91089	GLP6	3.6595764	Germin-like protein, putative
A_84_P20216	AT3G05950	9.693682	RPP4 (recognition of peronosp para)
A_84_P257040	RPP4	1.6975852	LCR44 (cysteine-rich 44)
A_84_P761249	LCR44	3.0183408	ATMRP11; ATPase
A_84_P14474	ATMRP11	1.9051145	ATATH7; ATPase
A_84_P10861	ATATH7	2.0049782	ABC transporter family protein
A_84_P21738	AT3G28345	2.91361	ProT3 (PROLINE TRANSPORTER 3)
A_84_P169313	ProT3	2.3417182	ATCNGC5 (CYCL NUCL GAT CHAN)
A_84_P850149	ATCNGC5	1.5476626	PIP1;4 (PLASMA MEMB INTR PROT)
A_84_P856515	PIP1;4	1.646188	PIP2E (PLASMA MEMB INTR PROT)
A_84_P850691	PIP2E	3.5367277	MATE efflux family protein

A_84_P549035	AT2G04090	1.6247929	VATG3 (vacuolar ATP synthase G3)
A_84_P11009	VATG3	1.7398367	SKOR; cyclic nucleotide binding
A_84_P23059	SKOR	2.608436	Anion exchange family protein
A_84_P795021	AT3G62270	3.13765	MISCRNA
A_84_P765653	AT4G16892	1.5244622	-
A_84_P826767	-	1.570697	-
A_84_P715149	-	1.6198127	MAF1 (MADS AFFE FLOWER)
A_84_P852212	MAF1	1.6276212	rRNA
A_84_P756698	AT2G01020	1.6507446	rRNA
A_84_P855375	AT2G01010	1.7716572	-
A_84_P808174	-	1.8372042	-
A_84_P797855	-	1.8506	BLH1 (BEL1-LIKE HOME DOM)
A_84_P852335	BLH1	1.8523728	-
A_84_P23043	-	1.913805	-
A_84_P803322	-	1.9208417	-
A_84_P862970	-	2.7801766	5733272 CERES-147
A_84_P796180	RKF3	2.8147972	AV440309 (above-ground organ)
A_84_P801290	GAPA	2.81844	Rep: Copper/zinc supero dis precursor
A_84_P856707	-	2.8412035	AT5G19900 mRNA
A_84_P827422	AT5G19900	3.0343535	AYBJU78TR pooled cDNA pop
A_84_P798944	CYP71B5	3.1188323	Rep: 60S ribosomal protein
A_84_P796287	-	5.377286	(Mouse-ear cress), complete
A_84_P853531	-	8.155037	